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(54) Title: HEAT SHOCK/STRESS PROTEIN COMPLEXES AS VACCINES AGAINST NEURODEGENERATIVE DISORDERS

(57) Abstract: The present invention relates to pharmaceutical compositions comprising complexes of heat shock proteins (hsps) in association with antigenic molecules for use in treatment and prevention of neurodegenerative disorders and disease. The invention further relates to methods for the use of such pharmaceutical compositions as immunotherapeutic agents for the treatment and prevention of neurodegenerative disorders and disease.

HEAT SHOCK/STRESS PROTEIN COMPLEXES AS VACCINES AGAINST NEURODEGENERATIVE DISORDERS

This invention was made with government support under grant numbers CA44786 and CA64394 awarded by the National Institutes of Health. The government has certain rights in the invention.

1. INTRODUCTION

The present invention relates to compositions and methods for the use of complexes of heat shock proteins and antigenic peptides associated with neurodegenerative disorders, which can be used as vaccines against neuropsychiatric disorders, such as Alzheimer's Disease.

2. BACKGROUND OF THE INVENTION

2.1 Neurodegenerative Disorders

Neurodegenerative disorders are typically characterized by a number of neuropathological abnormalities, such as neuritic plaques, neurofibrillary tangles (NFTs), Lewy bodies, and intranuclear inclusions. For example, Alzheimer's Disease, the most common cause of dementia, is characterized pathologically by neurodegeneration, the presence of large numbers of amyloid plaques, and neurons that accumulate tau and ubiquitin reactivities within NFTs. The result is damage to regions of the brain and neural circuits responsible for memory and cognition, including neurons in the neocortex, hippocampus, amygdala, basal forebrain cholinergic system, and brainstem monoaminergic nuclei.

Neuritic plaques are extracellular lesions, consisting largely of a deposit of a peptide called β-amyloid (Aβ). The predominant forms of β-amyloid are the 40 amino-acid form, Aβ40, and the 42 amino acid form, Aβ42. Most secreted forms of Aβ peptides are Aβ40, a soluble species, whereas about 10% of secreted Aβ peptides are Aβ42 and Aβ43, insoluble forms of the peptide that are highly fibrillogenic and deposited in plaques. The plaques in the brains of AD patients are composed predominantly of these fibrillar forms of Aβ. Full-length synthetic Aβs [Aβ-(1-40) and Aβ-(1-42)] and Aβ-(25-35) also form fibrils and are neurotoxic (see, for example, Howlett *et al.*, 1995, Neurodegeneration 4: 23-32; Pike *et al.*, 1993, J. Neuroscience 13: 1676-1687; Yankner *et al.*, 1990, Science 250: 279-282).

Neurofibrillary tangles are intracellular lesions consisting of poorly soluble filaments of the protein tau. In particular, hyperphosphorylated forms and highly ubiquitinated forms of the tau protein, a microtubule-binding protein, are predominant in such NFTs. Tangles are found in frontotemporal dementia associated with Parkinson's disease, Alzheimer's Disease, progressive supranuclear palsy, Guam disease, and some forms of prion disease. Lewy bodies, consisting largely of the protein α-synuclein, are found in Parkinson's disease, some forms of Alzheimer's disease, and Lewy body dementia (for review, see Hardy and Hardy, 1998, Science 282: 1075-1079 and Greefnfield's Neuropatholgy, Graham and Lantos (eds.), Arnold, London, 1997).

Intranuclear inclusions are often associated with an unstable triplet repeat mutation (polyCAG) which cause polyglutamine diseases. The presence of the repeat, which encodes polyglutamine, in the gene encoding huntingtin, has been shown to cause intranuclear polyglutamine inclusions which typify Huntington's Disease (Davies *et al.*, 1997, Cell 90: 537; Davies *et al.*, 1998, Lancet 351: 131)

Neuropsychiatric and neurodegenerative disorders are beginning to be understood at the molecular level. For example, mutations or polymorphisms in four genes are known that give rise to an increased risk of, or early onset of, AD: amyloid precursor protein (APP), the precurser to Aβ (Goate *et al.*, 1991, Nature 349: 704-706); an allele of apolipoprotein, ApoE4; presenilin-1 (PS1; Sherrington *et al.*, 1995, Nature 375: 754-760) ; and presenilin-2 (PS2; Levy-Lahad *et al.*,1995, Science 269: 973-977). Mutations in each of these four genes can have the same result -- an increased production of the 42-43 amino acid form of Aβ. APP, a single transmembrane domain integral membrane glycoprotein, is processed via alternative proteolytic pathways. In one pathway, APP molecules are cleaved within the Aβ sequence by a plasma membrane protease, resulting in secretion of the ectodomain of APP. In an alternative pathway, Aβ peptides are generated by endoproteolytic cleavage of APP. Mutations in APP can result in the shift of the processing of the APP precurser so that more of the 42 or 43 amino acid form of Aβ is produced. PS1 and PS2 are highly homologous 43 to 50 kD proteins with eight transmembrane domains. Presenilin polypeptides acculmulate as 27/28 kDa N-terminal and 16/17 kDa C-terminal kDa derivatives that become stably associated with each other *in vivo*. Most genetic abnormalities in the presenilin are missense mutations that result in single amino acid substitutions. Unlike the case of early onset of AD, no specific gene mutations are associated with the inheritance in the case of late onset AD. However, specific alleles of apolipoprotein E and α-2 macroglobulin are associated with increased risk for AD (Blacker *et al.*, 1998, Nature Genet. 19: 357-360).

Likewise, the molecular basis of neurodegenerative diseases other than AD are also beginning to be understood. Strikingly similar pathologies commonly associated with the neurodegenerative disorders, can be arrived at by a large number of different genetic mechanisms. For example, a pathogenic mutation in the prion gene results in both tangle and Lewy body pathologies of prion disease (Feany and Kickson, 1995, Am. J. Pathol. 146: 1388). Mutations in tau protein lead to NFTs and dementia in frontotemporal dementia (Hutton et al., 1998, Nature 393: 702), mutations in synuclein lead to the presence of Lewy bodies and Parkinson's disease (Polymeropoulos *et al.*, 1997, Science 276: 2045).

2.2 Heat Shock Proteins

Heat shock proteins (hsps), also referred to interchangeably as stress proteins, were first identified as proteins synthesized by a cell in response to heat shock. To date, five major classes of hsps have been identified, based on the molecular weight of the family members. These classes are called shsps (small heat shock proteins), Hsp60, Hsp70, Hsp90, and Hsp100, where the numbers reflect the approximate molecular weight of the hsps in kilodaltons.

Many hsps have been found to be induced in response to stressful stimuli other than heat, including nutrient deprivation, metabolic disruption, oxygen radicals, and infection with intracellular pathogens (see Welch, May 1993, Scientific American 56-64; Young, 1990, Annu. Rev. Immunol. 8: 401-420; Craig, 1993, Science 260: 1902-1903; Gething *et al.*, 1992, Nature 355: 33-45; and Lindquist *et al.*, 1988, Annu. Rev. Genetics 22: 631-677). Heat shock proteins are highly conserved proteins. For example, DnaK, the Hsp70 from E. coli has about 50% amino acid sequence identity with Hsp70 proteins from excoriates (Bardwell *et al.*, 1984, Proc. Natl. Acad. Sci. U.S.A. 81: 848-852). The Hsp60 and Hsp90 families also show similarly high levels of intra-family conservation (Hickey *et al.*, 1989, Mol. Cell. Biol. 9: 2615-2626; Jindal, 1989, Mol. Cell. Biol. 9: 2279-2283).

Hsps are involved not only in cellular protection against adverse conditions, but are also involved in essential biochemical and immunological processes in unstressed cells. For example, hsps are involved in various kinds of chaperoning functions. Members of the Hsp70 family, located in the cell cytoplasm, nucleus, mitochondria, or endoplasmic reticulum are involved in the presentation of antigens to the cells of the immune system, and are also involved in the transfer, folding and assembly of proteins in normal cells (Lindquist *et al.*, 1988, Ann. Rev. Genetics 22: 631-677). A number of proteins thought to be involved in chaperoning are residents of the endoplasmic reticulum (ER) lumen, for example, protein disulfide isomerase (PDI; Gething *et al.*, 1992, Nature 355: 33-45), Grp94 or ERp99 (Sorger & Pelham, 1987, J. Mol. Biol. 194: 991-94) which is related to Hsp90,

and Grp78 or BiP, which is related to Hsp70 (Munro *et al.*, 1986, Cell 46: 291-300; Haas & Webl, 1983, Nature 306: 387-389). These proteins are known to bind a variety of mutant, unfolded, incompletely glycosylated proteins (Machamer *et al.*, 1990, J. Biol. Chem.. 65: 6879-6883; Gething *et al.*, 1986, Cell 46: 939-950).

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2.3 Immunogenicity of Heat Shock/Stress Proteins

Srivastava *et al.* demonstrated immune response to methylcholanthrene-induced sarcomas of inbred mice (Srivastava *et al.*, 1988, Immunol. Today 9: 78-83). In these studies, it was found that the molecules responsible for the individually distinct
10 immunogenicity of these tumors were cell-surface glycoproteins of 96kDa (gp96) and intracellular proteins of 84 to 86kDa (Srivastava *et al.*, 1986, Proc. Natl. Acad. Sci. U.S.A. 83: 3407-3411; Ullrich *et al.*, 1986, Proc. Natl. Acad. Sci. U.S.A. 83: 3121-3125). Immunization of mice with gp96 or p84/86 isolated from a particular tumor rendered the mice immune to that particular tumor, but not to antigenically distinct tumors. Isolation and
15 characterization of genes encoding gp96 and p84/86 revealed significant homology between them, and showed that gp96 and p84/86 were, respectively, the endoplasmic reticular and cytosolic counterparts of the same heat shock proteins (Srivastava *et al.*, 1988, Immunogenetics 28: 205-207; Srivastava *et al.*, 1991, Curr. Top. Microbiol. Immunol. 167: 109-123). Further, Hsp70 was shown to elicit immunity to the tumor from which it was
20 isolated but not to antigenically distinct tumors. However, Hsp70 depleted of peptides was found to lose its immunogenic activity (Udono and Srivastava, 1993, J. Exp. Med. 178: 1391-1396). These observations suggested that the heat shock proteins are not immunogenic per se, but form noncovalent complexes with antigenic peptides, and the complexes can elicit specific immunity to the antigenic peptides (Srivastava, 1993, Adv.
25 Cancer Res. 62: 153-177; Udono *et al.*, 1994, J. Immunol. 152: 5398-5403; Suto *et al.*, 1995, Science 269: 1585-1588).

The use of noncovalent complexes of stress proteins and peptides, purified from cancer cells, for the treatment and prevention of cancer, as well as the use of such complexes in combination with adoptive immunotherapy, has been described (see U.S.
30 Patent No. 5,750,199; U.S. Patent No. 5,830,464; Patent Cooperation Treaty ("PCT") publications WO 96/10411, dated April 11, 1996; and WO 97/10001, dated March 20, 1997), each of which is incorporated by reference herein in its entirety. The purification of stress protein-peptide complexes from cell lysates has been described previously; stress protein-peptide complexes can be isolated from pathogen-infected cells and used for the
35 treatment and prevention of infection caused by pathogens, such as viruses and other

intracellular pathogens, including bacteria, protozoa, fungi and parasites (see PCT publication WO 95/24923, dated September 21, 1995).

Immunogenic stress protein-peptide complexes can also be prepared by *in vitro* complexing of stress protein and antigenic peptides, and the uses of such complexes for the treatment and prevention of cancer and infectious diseases has been described in PCT publication WO 97/10000, dated March 20, 1997. The use of heat shock proteins in combination with a defined antigen for the treatment of cancer and infectious diseases have also been described in PCT publication WO 97/06821, dated February 27, 1997. The administration of expressible polynucleotides encoding eukaryotic heat shock proteins to mammalian cells for stimulating an immune response, and for treatment of infectious diseases and cancer has been described in PCT publications, WO 97/06685 and WO 97/06828, both dated February 27, 1997. The use of stress protein-peptide complexes for sensitizing antigen presenting cells *in vitro* for use in adoptive immunotherapy is described in PCT publication WO 97/10002, dated March 20, 1997.

These references, however, have not described the use of complexes of heat shock proteins and defined antigenic peptides as vaccines for treatment or prevention of a neurodegenerative disorder. Vaccination has been used against infectious diseases such as polio, tetanus, chicken pox, measles, *etc.*, and has eradicated these diseases in many countries of the world. Such vaccination with non-live materials such as proteins generally leads to an antibody response or CD4+ helper T cell response (Raychaudhuri and Morrow, 1993, Immunology Today 14: 344-348). On the other hand, vaccination or infection with live materials such as live cells or infectious viruses generally leads to a CD8+ cytotoxic T-lymphocyte (CTL) response. Methods for the use of complexes of heat shock proteins and antigenic peptides as vaccines to generate an immune response against such neurodegenerative disorders would greatly increase the prospects of treating and preventing such disorders.

Citation or discussion of a reference herein shall not be construed as an admission that such is prior art to the present invention.

3. SUMMARY OF THE INVENTION

The present invention provides methods for the use of complexes of heat shock proteins and antigenic peptides as vaccines for the treatment and prevention of neurodegenerative disorders, such as Alzheimer's Disease.

In one aspect of the invention, a purified complex is provided comprising a heat shock protein and an antigenic molecule, which antigenic molecule displays the

antigenicity of an antigen associated with a neurodegenerative disorder. In one embodiment, the heat shock protein is noncovalently associated with the antigenic molecule. In another embodiment, the heat shock protein is noncovalently associated with the antigenic molecule. In another embodiment, the complex of a heat shock protein and an antigenic molecule is purified to apparent homogeneity, as viewed on an SDS-PAGE gel. In another embodiment, the complex is the product of a method comprising complexing said heat shock protein and said antigenic molecule in vitro. In another embodiment, the complex is a fusion protein comprising the heat shock protein fused to the antigenic molecule. In another embodiment, the heat shock protein is Hsp70, Hsp90, gp96, PDI, or calreticulin. In another embodiment, the heat shock protein is covalently associated with the antigenic molecule. In another embodiment, the antigenic molecule is β-amyloid or a fragment thereof, an oligomeric Aβ complex or a fragment thereof, an ApoE4-Aβ complex or a fragment thereof, tau protein or a fragment thereof, a mutant amyloid precurser protein or a fragment thereof, a mutant of presenillin or a fragment thereof, α-synuclein or a fragment thereof, or a prion protein or a fragment thereof. In a specific embodiment, the antigenic molecule is β-amyloid, or a fragment thereof.

In another embodiment, the invention provides a composition comprising a purified population of complexes of heat shock proteins bound to antigenic molecules, said complexes being purified from cells transformed with and expressing a nucleic acid encoding a protein displaying the antigenicity of an antigen associated with a neurodegenerative disorder. In another embodiment, the population of complexes of heat shock proteins bound to antigenic molecules is purified to apparent homogeneity, as viewed on an SDS-PAGE gel. Preferably, the ND-associated hsp-peptide complexes of the invention are used in purified form, preferably to apparent homogeneity as viewed on an SDS-PAGE gel, or to at least 60%, 70%, 80%, or 90% of total protein.

In another aspect of the invention, a pharmaceutical composition is provided comprising an amount of a purified molecular complex effective for treatment or prevention of a neurodegenerative disorder, and a pharmaceutically acceptable carrier, said molecular complex comprising a heat shock protein and an antigenic molecule, which antigenic molecule displays the antigenicity of an antigen associated with a neurodegenerative disorder. In one embodiment, the heat shock protein is covalently associated with the antigenic molecule. In another embodiment, the antigenic molecule is coupled to a universal helper. In another embodiment, the heat shock protein is Hsp70, Hsp90, gp96, PDI, or calreticulin. In another embodiment, the antigenic molecule is β-amyloid or a fragment thereof, an oligomeric Aβ complex or a fragment thereof, an ApoE4-Aβ complex or a fragment thereof, tau protein or a fragment thereof, a mutant amyloid precurser protein or a fragment thereof, a mutant of presenillin or a fragment thereof, α-synuclein or a

fragment thereof, or a prion protein or a fragment thereof. In a specific embodiment, the antigenic molecule is β-amyloid, or a fragment thereof.

The invention further provides a recombinant cell transformed with (i) a first nucleic acid comprising a first nucleotide sequence that is operably linked to a first promoter and that encodes a heat shock protein, and (ii) a second nucleic acid comprising a second nucleotide sequence that is operably linked to a second promoter and encodes an antigenic molecule that displays the antigenicity of an antigen associated with a neurodegenerative disorder, such that the heat shock protein and the antigenic molecule are expressed within the cell and non-covalently associate with each other to form a complex that in sufficient amount is capable of eliciting an immune response to the antigenic molecule. In one embodiment, the cell is a human cell. In another embodiment, the antigenic molecule is β-amyloid or a fragment thereof, an oligomeric Aβ complex or a fragment thereof, an ApoE4-Aβ complex or a fragment thereof, tau protein or a fragment thereof, a mutant amyloid precurser protein or a fragment thereof, a mutant of presenillin or a fragment thereof, α-synuclein or a fragment thereof, or a prion protein or a fragment thereof. In a specific embodiment, the antigenic molecule is β-amyloid, or a fragment thereof.

In another aspect of the invention, the invention provides a recombinant cell transformed with a nucleic acid comprising a nucleic acid sequence that is operably linked to a promoter, said nucleic acid sequence encoding a fusion protein that comprises a heat shock protein operatively linked to an antigenic molecule that displays the antigenicity of an antigen associated with a neurodegenerative disorder. In one embodiment, the cell is a human cell. In another embodiment, the antigenic molecule is β-amyloid or a fragment thereof, an oligomeric Aβ complex or a fragment thereof, an ApoE4-Aβ complex or a fragment thereof, tau protein or a fragment thereof, a mutant amyloid precurser protein or a fragment thereof, a mutant of presenillin or a fragment thereof, α-synuclein or a fragment thereof, or a prion protein or a fragment thereof. In a specific embodiment, the antigenic molecule is β-amyloid, or a fragment thereof.

In one embodiment, the invention provides pharmaceutical compositions comprising recombinant cells and pharmaceutically acceptable carriers.

The invention further provides a method for preparing a complex of a heat shock protein associated with an antigenic molecule that displays the antigenicity of an antigen associated with a neurodegenerative disorder, comprising: a) culturing a recombinant cell that expresses a heat shock protein and is transformed with a nucleic acid comprising a nucleotide sequence that is operably linked to a promoter and encodes an antigenic molecule that displays the antigenicity of an antigen associated with a neurodegenerative disorder, under conditions such that the antigenic molecule is expressed

by the cell and associates with the heat shock protein expressed by the cell; and b) recovering a population of complexes of the heat shock proteins noncovalently associated with the antigenic molecules. In one embodiment, the method further comprises the step of c) purifying the complexes. In a specific embodiment of this method, the complexes are purified by affinity chromatography. In another embodiment, the method further comprises the step of d) treating the purified complexes with a crosslinking agent such that the hsps become covalently attached to the antigenic molecules.

In another embodiment, a method for preparing a complex of a heat shock protein associated with an antigenic molecule that displays the antigenicity of an antigen associated with a neurodegenerative disorder is provided, comprising: a) culturing a recombinant cell transformed with (i) a first nucleic acid comprising a first nucleotide sequence that is operably linked to a first promoter and that encodes a heat shock protein, and (ii) a second nucleic acid comprising a second nucleotide sequence that is operably linked to a second promoter and encodes an antigenic molecule that displays the antigenicity of an antigen associated with a neurodegenerative disorder, under conditions such that the heat shock protein is expressed by the cell and associates with the antigenic molecule; and b) recovering a population of complexes of the heat shock protein noncovalently associated with antigenic molecules from the cells. In one embodiment, the method further comprises the step of c) purifying the complexes. In a specific embodiment of this method, the complexes are purified by affinity chromatography. In another embodiment, the method further comprises the step of d) treating the purified complexes with a crosslinking agent such that the hsps become covalently attached to the antigenic molecules.

In another aspect of the invention, a method is provided for preparing a fusion protein capable of eliciting an immune response against a neurodegenerative disorder, said method comprising: a) culturing a recombinant cell transformed with a nucleic acid comprising a nucleotide sequence that is operably linked to a promoter and that encodes a fusion protein comprising a heat shock protein operatively linked to an antigenic molecule that displays the antigenicity of an antigen associated with a neurodegenerative disorder, under conditions such that the fusion protein is expressed by the cell; and b) recovering the fusion protein from the cells.

The invention further provides methods for preparing *in vitro* complexes of heat shock proteins associated with one or more ND-associated antigenic molecules. In one embodiment, a method for preparing *in vitro* complexes of heat shock proteins associated with one or more ND-associated antigenic molecules is provided, said method comprising: a) incubating a heat shock protein and one or more antigenic molecules under conditions and for a length of time sufficient for the formation of the complexes, wherein said

antigenic molecules display the antigenicity of an antigen associated with a neurodegenerative disorder, and b) isolating said complexes. In one embodiment, the heat shock protein is purified. In one embodiment, the method further comprises the step of c) treating the isolated complexes with a crosslinking agent such that the hsp of the complex becomes covalently attached to one or more antigenic molecule of the complex.

The antigenic molecules used in the methods of the invention display the antigenicities of antigens associated with a neurodegenerative disorder. In various embodiments, one or more of the antigenic molecules are β-amyloid or a fragment thereof, an oligomeric Aβ complex or a fragment thereof, an ApoE4-Aβ complex or a fragment thereof, tau protein or a fragment thereof, a mutant amyloid precurser protein or a fragment thereof, a mutant of presenillin or a fragment thereof, α-synuclein or a fragment thereof, or a prion protein or a fragment thereof. In a specific embodiment, one or more of the antigenic molecules is β-amyloid, or a fragment thereof.

In one embodiment, a method is provided for eliciting an immune response against an antigen associated with a neurodegenerative disorder in an individual comprising administering to the individual a complex of a heat shock protein and an antigenic molecule that displays the antigenicity of an antigen associated with a neurodegenerative disorder in an amount effective to elicit an immune response. In one embodiment the method further comprises, before, concurrently, or after administration of the immunogenic complex, administering to the individual a composition comprising antigen presenting cells sensitized *in vitro* with a sensitizing amount of a second complex of a heat shock protein and a second antigenic molecule, in which said second antigenic molecule shares at least one antigenic determinant with the first antigenic molecule.

In another embodiment, the invention provides a method of treating or protecting against a neurodegenerative disorder in an individual having a neurodegenerative disorder, or in whom prevention of a neurodegenerative disorder is desired, comprising administering to the individual a purified complex of a heat shock protein and an antigenic molecule in an amount effective to treat or protect against said neurodegenerative disorder, wherein said antigenic molecule displays the antigenicity of an antigen associated with said neurodegenerative disorder. In one embodiment, the method, further comprises, before, concurrently or after administration of the immunogenic complex, administering to the individual a composition comprising antigen presenting cells sensitized *in vitro* with a sensitizing amount of a second complex of a heat shock protein and a second antigenic molecule, said second antigenic molecule sharing at least one antigenic determinant with the first antigenic molecule.

In one embodiment, the invention provides a method of treating or protecting against a neurodegenerative disorder in a subject having a neurodegenerative disorder or in

whom prevention of a neurodegenerative disorder is desired comprising: a) culturing a recombinant cell that expresses a heat shock protein and is transformed with a nucleic acid comprising a nucleotide sequence that is operably linked to a promoter and encodes an antigenic molecule that displays the antigenicity of an antigen associated with a neurodegenerative disorder, under conditions such that the antigenic molecule is expressed by the cell and associates with the heat shock protein expressed by the cell; and b) recovering a population of complexes of the heat shock proteins noncovalently associated with the antigenic molecules; and c) administering to the subject an amount of the recovered complexes effective to treat or protect against the neurodegenerative disorder. In one embodiment, the method further comprises, after step (b) and before step (c), the step of treating the complexes with a crosslinking agent such that the heat shock protein becomes covalently associated with the antigenic molecule.

In another embodiment, the invention provides a method of treating or protecting against a neurodegenerative disorder in a subject having a neurodegenerative disorder or in whom prevention of a neurodegenerative disorder is desired comprising:a) culturing a recombinant cell transformed with (i) a first nucleic acid encoding a heat shock protein, and (ii) a second nucleic acid encoding an antigenic molecule displaying the antigenicity of an antigen associated with a neurodegenerative disorder; b) recovering complexes of the heat shock protein noncovalently associated with the antigenic molecule; and c) administering to the subject an amount of the recovered complexes effective to treat or protect against the neurodegenerative disorder. In one embodiment, the method further comprises, after step (b) and before step (c), the step of treating the complexes with a crosslinking agent such that the heat shock protein becomes covalently associated with the antigenic molecule.

In another embodiment, the invention provides a method of treating or protecting against a neurodegenerative disorder in a subject having a neurodegenerative disorder or in whom prevention of a neurodegenerative disorder is desired comprising: a) culturing a recombinant cell transformed with a nucleic acid comprising a nucleotide sequence that is operably linked to a promoter and that encodes a fusion protein comprising a heat shock protein operatively linked to an antigenic molecule that displays the antigenicity of an antigen associated with a neurodegenerative disorder, under conditions such that the fusion protein is expressed by the cell; b) recovering the fusion protein from the cells; and c) administering to the subject an amount of the fusion protein effective to treat or protect against the neurodegenerative disorder.

Neurodegenerative disorders that can be prevented or treated using the compositions and methods of the invention include disorders relating to the central nervous system and/or peripheral nervous system including, but not limited to, cognitive and

neurodegenerative disorders such as Alzheimer's Disease, age-related loss of cognitive function and senile dementia, Parkinson's disease, amyotrophic lateral sclerosis, Wilson's Disease, cerebral and progressive supranuclear palsy, Guam disease, Lewy body dementia, prion diseases, such as spongiform encephalopathies, *e.g.*, Creutzfeldt-Jakob disease, polyglutamine diseases, such as Huntington's disease, myotonic dystrophy, Freidrich's ataxia and other ataxias, well as Gilles de la Tourette's syndrome, seizure disorders such as epilepsy and chronic seizure disorder, stroke, brain or spinal cord trauma, AIDS dementia, alcoholism, autism, retinal ischemia, glaucoma, autonomic function disorders such as hypertension and sleep disorders, and neuropsychiatric disorders that include, but are not limited to schizophrenia, schizoaffective disorder, attention deficit disorder, dysthymic disorder, major depressive disorder, mania, obsessive-compulsive disorder, psychoactive substance use disorders, anxiety, panic disorder, as well as unipolar and bipolar affective disorders. Additional neuropsychiatric and neurodegenerative disorders include, for example, those listed in the American Psychiatric Association's Diagnostic and Statistical manual of Mental Disorders (DSM), the most current version of which is incorporated herein by reference in its entirety.

4. DETAILED DESCRIPTION OF THE INVENTION

The invention provides compositions of complexes of hsps and antigenic peptides associated with neurodegenerative diseases and disorders, such as Alzheimer's Disease (AD), which can be used as vaccines to protect against and/or treat such diseases and disorders.

In certain embodiments, the compositions and formulations of the present invention are administered to a human subject to prevent a neurodegenerative disorder (ND), including inhibiting the progression of a disease in an asymptomatic patient, for example a patient having the molecular landmarks of AD (*e.g.*, above normal levels of phosphorylated Tau and/or Aβ42). In a preferred embodiment, the human subject to which the vaccines of the invention are administered is one having a genetic background that increases the likelihood of a given ND (*e.g.* for Alzheimer's Disease, having the E4 allele of Apolipoprotein E or having a mutation in APP, PS1 or PS2 which gives rises to FAD). In another embodiment, the human subject to which the preventative vaccines of the invention are administered is a non-senile adult above the age of 60.

In other embodiments, the compositions and formulations of the present invention are administered to a human subject that has been diagnosed with a ND or suspected of having a ND. According to the present invention, treatment of a ND encompasses the treatment of patients already diagnosed as having ND at any clinical stage; the prevention of the disease in the patients with early symptoms and signs; the delay of the

onset or evolution or aggravation or deterioration of the symptoms or signs of a ND; and/or promoting regression of a ND in symptomatic patients.

Antigenic peptides that can be used to form such hsp-antigenic peptide complexes are peptides derived from material found in the brains of patients with neurodegenerative disorders, or synthesized based on the known sequences of peptides associated with the pathology of such disorders. Such peptides include, but are not limited to, peptides of, or derived from, the following: β-amyloids found in neurodegenerative plaques, for example, plaques associated with AD; tau protein, which is found in neurofibrillary tangles (NFTs); α-synuclein, which is found in Lewy bodies; prion protein (PrP), which is associated with spongiform encephalopathies, *e.g.*, Creutzfeldt-Jakob disease, and other peptides associated with such neuropsychiatric disorders. Such antigenic peptides are described in detail in Section 4.1, below.

Complexes between hsps and ND-associated peptide antigens (hsp-ND-associated peptide complexes) can be produced by any of a number of methods. For example, antigenic peptides associated with neurodegenerative disorders and hsps can be obtained by recombinant or synthetic methods, or can be isolated and purified from recombinant cells. Complexes between neurodegenerative disorder-associated peptides and hsps can be formed by covalent or non-covalent association of antigenic peptides with hsps. Preferably, the ND-associated hsp-peptide complexes of the invention are used in purified form, preferably to apparent homogeneity as viewed on an SDS-PAGE gel, or to at least 60%, 70%, 80%, or 90% of total protein.

In addition, complexes may be formed *in vivo* or *in vitro* using a variety of methods, described herein. Methods for preparing such hsp antigenic complexes are described in detail in Sections 4.2 to 4.4, below.

Heat shock protein-antigenic peptide complexes may be used as vaccines against neurodegenerative diseases and disorders. Without being bound by any particular theory, such complexes may act by eliciting a B-cell and/or T-cell response in patients with such disorders. As part of the B-cell response, complexes of hsps with ND-associated antigens may generate antibodies specific for neurodegenerative disease-associated peptides, which in turn, may accelerate clearance of antigenic materials that accumulate in the brains of patients with such diseases. As part of the T-cell response, complexes of hsps with antigenic peptides may elicit a cytotoxic T cell response against cells that express neurodegenerative disease-associated peptide sequences. Methods for the use of such hsp-neurodegenerative disease-associated peptide complexes as vaccines against such neurodegenerative disorders are described in Section 4.5 in detail herein.

4.1 Neurodegenerative Disease Antigens

Peptide antigens associated with neurodegenerative diseases and disorders (ND), herein termed "ND-associated antigens", comprise peptides and polypeptides, and fragments thereof, that are found associated with plaques, tissues or cells of subjects with neurodegenerative diseases and disorders, such as Alzheimer's Disease, and that are specific to subjects with such diseases and disorders. Such antigenic peptides, can be complexed with heat shock proteins and used to elicit an immune response against such disorders. ND-associated antigens include, but are not limited to, β-amyloid (Aβ) peptides and fragments thereof, oligomeric Aβ complexes and fragments thereof, fragments of ApoE4-Aβ complexes, hyperphosphorylated tau and fragments thereof, APP mutant proteins and fragments thereof, presenillin mutant proteins and fragments thereof, α-synuclein of Lewy bodies and fragments thereof, prion protein (PrP) and fragments thereof, as well as other antigenic peptides present in ND plaques, neurofibrillary tangles and lesions, cells, and tissues. Such antigenic ND-associated peptides may be produced by any synthetic or recombinant means known in the art. The compositions of such peptides and the methods for their production, isolation, or synthesis are described in detail hereinbelow.

4.1.1 ND-Associated Antigenic Molecules

Antigenic peptides associated with nueropsychiatric disease or neurodegenerative disorders, or antigenic portions thereof, can be chosen from among those known in the art to be associated with such diseases and disorders. Alternatively, such antigens can be selected for their antigenicity or their immunogenicity, as determined by immunoassays or by their ability to generate an immune response.

In a preferred embodiment, antigenic peptides may be derived from β-amyloid (Aβ). Peptide fragments of Aβ-(1-40) and Aβ-(1-42)] and Aβ-(25-35) are used. Full-length Aβ peptides [Aβ-(1-40) and Aβ-(1-42)] and Aβ-(25-35) form fibrils and are neurotoxic (see, for *e.g.*, Howlett *et al.*, 1995, Neurodegeneration 4: 23-32; Pike *et al.*, 1993, J. Neuroscience 13: 1676-1687; Yankner *et al.*, 1990, Science 250: 279-282). The predominant forms of β-amyloid are the 40 amino-acid form, Aβ40, and the 42 amino acid form, Aβ42. In particular, amino acids 35 (methionine) and 41-42 appear to play important roles in the process of fibril formation. Therefore, in this embodiment, a peptide antigen comprising Aβ40, or fragments thereof, comprising the amino acid sequence (N)DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV (SEQ ID NO:1), or a fragment comprising 5 to 10, 10 to 15, 15 to 20, 20 to 30, or 30 to 40 consecutive amino acids thereof may be used as an antigen. For example, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, or 39 consecutive amino acids thereof, can be used as antigenic peptides. In another embodiment, the 42 amino acid

form, Aβ42, comprising the amino acid sequence (N)DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA (SEQ ID NO:2), or a fragment containing 10, 11, 12, 13, 14, 15 15, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, or 41 consecutive amino-acids thereof, can be used as peptide antigens for use in hsp complexes as vaccines against AD.

Complexes of hsps with allelic variants of Aβ40 and Aβ42 are also within the scope of the invention. An allele is one of a group of genes which occur alternatively at a given genetic locus. As will be appreciated by those skilled in the art, DNA sequence polymorphisms that result in changes in amino acid sequence of an AD-associated antigen such as Aβ40 and Aβ42 will exist within a population of individual organisms (*e.g.*, within a human population). Such polymorphisms may exist, for example, among individuals within a population due to natural allelic variation.

In another embodiment, an antigen may comprise a dimeric, oligomeric or multimeric form of a β-amyloid. In particular, fibrils can be formed by *in vitro* polymerization of Aβ40 and Aβ42 (Harper and Lansbury, 1997, Annu. Rev. Biochem. 66: 385-407). The Aβ amyloid protofibril is a discrete intermediate in the *in vitro* process of Aβ fibril formation (Harper *et al.*, 1997, Chem. Biol. 4: 119-125). This stable intermediate, and proteolytic fragments thereof, can also be used as antigenic peptides for complexing with hsps.

In another embodiment, apolipoproteins, or fragments thereof, can be used as antigens. Apolipoprotein E (ApoE) is present in amyloid plaques in AD (Namba *et al.*, 1991, Brain Res. 541: 163-166). One isoform of ApoE, ApoE4, is particularly associated with a high risk for AD. Thus, complexes of ApoE4, or derivatives, fragments, or analogs thereof, can be sources for antigenic peptides. In addition, complexes of apolipoproteins and Aβ, or fragments such as proteolytic products thereof, can also be used as antigens.

In various embodiments, antigenic peptides may comprise amino acid sequences derived from APP proteins known to be associated with AD, or fragments thereof. In a specific embodiment, peptide fragments of a mutant APP comprising a mutation at codon 717 may be used (Chartier-Harlin *et al.*, 1991, Nature 353: 844-6). In another specific embodiment, antigenic molecules may be peptide fragments of a mutant APP comprising a mutation at codon 670 or 671.

In another embodiment, antigenic peptides derived from tau protein sequences are used. Hyperphosphorylated and ubiquitinated forms of the microtubule-associated protein tau, as well as tau mutations, are found associated with the pathologies of many neurodegenerative disorders, including AD (Lynch *et al.*, Neurology 44: 1878-1884; Spillantini *et al.*, 1998, Proc. Natl. Acad. Sci. U.S.A. 95: 7737-41). Thus, tau protein-derived antigenic peptides may be used to elicit an immune response against such

neurodegenerative disorders. The gene sequence encoding tau protein and the corresponding proteins sequences are available in public databases (GenBank accession no. NM_005910). In addition, modified amino acid sequences derived from hyperphosphorylated or ubiquitinated tau protein are also within the scope of the invention.

In another embodiment, antigenic peptides may comprise amino acid sequences derived from presenilin mutant proteins, PS1 (GenBank accession no. NM_000021; Sherrington *et al.*, 1995, Nature 375: 754-760) and PS2 (GenBank accession no. 000447 ; Levy-Lahad *et al.*, 1995, Science 269: 973-977).

In another embodiment, antigenic peptides may comprise amino acid sequences derived from α-synuclein (GenBank accession no. AF044672), which is found in Lewy bodies in various neurodegenerative disorders. Nucleotide sequences encoding α-synuclein amino acid sequences may be used to for recombinant expression of antigenic α-synuclein peptide fragments. Such α-synuclein antigenic molecules may be used to treat a subject with a neurodegenerative disorder having the pathology of the presence of Lewy bodies. Such disorders include, but are not limited to, Parkinson's disease, some forms of Alzheimer's disease, and Lewy body dementia.

In another embodiment, antigenic peptides may comprise amino acid sequences derived from the known amino acid sequence of prion protein (PrP; GenBank accession no. AF076976; GenBank accession no. NM_000311, Kretzschmar *et al.*, 1986, DNA 5: 315-324). Such prion antigenic molecules may be used to treat a subject with a neurodegenerative disorder having a prion pathology. Such disorders include, but are not limited to, spongiform encephalopathies, *e.g.*, Creutzfeldt-Jakob disease.

In another embodiment, antigenic peptides may comprise amino acid sequences derived from the huntingtin protein sequences (GenBank accession no. NM_002111), and mutants, variants or fragments thereof. Such antigenic peptides can be used to form antigenic complexes with hsps to treat subjects with Huntington's Disease, or any other disease associated with altered activity or expression of huntingtin. In another embodiment, polyglutamine repeats, as well as proteins containing polyglutamine repeats are used as antigenic peptides.

If the amino acid sequences of an ND-associated antigenic peptide is already not known, it may be determined either by manual or automated amino acid sequencing techniques well known in the art. Amino acid sequences and nucleotide sequences of antigenic molecules described above are generally available in sequence databases, such as GenBank. Computer programs, such as Entrez, can be used to browse the database, and retrieve any amino acid sequence and genetic sequence data of interest by accession number. These databases can also be searched to identify sequences with various degrees of

similarities to a query sequence using programs, such as FASTA and BLAST, which rank the similar sequences by alignment scores and statistics.

4.1.2. ND-Associated Antigen-T Cell Epitope Fusions

To increase the immunogenicity of an ND-associated antigenic peptide, the ND-associated antigenic peptide may be coupled to a carrier protein comprising one or more T cell epitopes. Such "universal" T helper cell epitopes can enhance immunogenicity of peptide constructs containing a limited number of epitopes. For example, because mycobacteria can induce a strong T-cell response, mycobacteria derivatives have been used to potentiate immune responses, with or without adjuvants (see Lachmann *et al.*, eds., 1986, in *Ciba Foundation Symposium on Synthetic Peptides as Antigens*, Wiley, Chichester, Vol. 119, pp. 25-57; and Lussow *et al.*, 1990, Proc. Natl. Acad. Sci. U.S.A. 87: 2960-2964). In one embodiment, antigens can be conjugated to a T helper cell epitope, or carrier protein, and used to immunize subjects, optionally primed with BCG (bacillus Calmette-Guerin *Mycobacterium tuberculosis* var. *bovis*). In a specific embodiment, such a carrier protein is tuberculin purified protein derivative (PPD). In another embodiment, tetanus toxin sequences can be used as carrier proteins, for example, tt830-844 from tetanus toxin (Kumar *et al.*, 1992, J. Immunol. 148: 1499-1505). In yet another embodiment, the circumsporozoite protein CST3 of a human malarial parasite, *Plasmodium falciparum* (Kumar *et al.*, supra), may be coupled to an ND-associated antigen to enhance immunogenicity.

The ND-associated antigenic peptide coupled to a universal helper comprising one or more T cell epitopes may then be complexed with heat shock proteins, as described in Section 4.3, below.

4.1.3 Synthetic Production of ND-Associated Antigens

Once the sequence of an ND-associated peptide antigen has been determined or obtained, the peptide can be produced, either by recombinant techniques or by synthetic methods. The antigenic peptide may be synthesized using conventional peptide synthesis or any of a number of other protocols well known in the art. For example, a peptide corresponding to a mutant protein associated with AD, such as an APP or presenilin mutant, can be synthesized by use of a peptide synthesizer. Either the entire protein can be synthesized, or an antigenic determinant thereof, preferably the portion of the protein that contains the mutant or variant amino acid(s).

An ND-associated peptide potentially useful for a vaccine protective against neurodegenerative diseases and disorders may be synthesized by using conventional peptide synthesis or other protocols well known in the art. Peptides having the same amino acid

sequence as peptides associated with ND-associated diseases and disorders may be synthesized by solid-phase peptide synthesis using procedures similar to those described by Merrifield, 1963, J. Am. Chem. Soc. 85: 2149. During synthesis, N-α-protected amino acids having protected side chains are added stepwise to a growing polypeptide chain linked by its C-terminal and to an insoluble polymeric support *i.e.*, polystyrene beads. The peptides are synthesized by linking an amino group of an N-α-deprotected amino acid to an α-carboxyl group of an N-α-protected amino acid that has been activated by reacting it with a reagent such as dicyclohexylcarbodiimide. The attachment of a free amino group to the activated carboxyl leads to peptide bond formation. The most commonly used N-α-protecting groups include Boc which is acid labile and Fmoc which is base labile. Details of appropriate chemistries, resins, protecting groups, protected amino acids and reagents are well known in the art (see Atherton *et al.*, 1989, Solid Phase Peptide Synthesis: A Practical Approach, IRL Press, and Bodanszky, 1993, Peptide Chemistry, A Practical Textbook, 2nd Ed., Springer-Verlag).

In addition, analogs and derivatives of ND-associated antigenic peptides can be chemically synthesized. Furthermore, if desired, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into the sequence of the ND-associated antigenic peptides. Non-classical amino acids include but are not limited to the D-isomers of the common amino acids, α-amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid, γ-Abu, ε-Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β-alanine, fluoro-amino acids, designer amino acids such as β-methyl amino acids, Cα-methyl amino acids, Nα-methyl amino acids, and amino acid analogs in general.

Purification of the resulting peptides is accomplished using conventional procedures, such as preparative HPLC using gel permeation, partition and/or ion exchange chromatography. The techniques, choice of appropriate matrices and buffers are well known in the art (Atherton *et al.*, 1989, Solid Phase Peptide Synthesis: A Practical Approach, IRL Press).

4.1.4 Recombinant Production of ND-Associated Antigens

As an alternative to synthetic production, ND-associated antigenic peptides and polypeptides, such as those mentioned in Section 4.1.1, above, can produced by recombinant means. Once the nucleotide sequence encoding an ND-associated antigen has been identified, the nucleotide sequence, or a fragment thereof, can be obtained and cloned into an expression vector for recombinant expression. The expression vector can then be

introduced into a host cell for propogation of the antigen. Methods for recombinant production of ND-associated peptide antigens are described in detail herein.

The DNA may be obtained by DNA amplification or molecular cloning directly from a tissue, cell culture, or cloned DNA (*e.g.*, a DNA "library") using standard molecular biology techniques (see *e.g.*, Methods in Enzymology, 1987, volume 154, Academic Press; Sambrook *et al.* 1989, Molecular Cloning - A Laboratory Manual, 2nd Edition, Cold Spring Harbor Press, New York; and Current Protocols in Molecular Biology, Ausubel et al. (eds.), Greene Publishing Associates and Wiley Interscience, New York, each of which is incorporated herein by reference in its entirety). Clones derived from genomic DNA may contain regulatory and intron DNA regions in addition to coding regions; clones derived from cDNA will contain only exon sequences. Whatever the source, the ND-associated antigen gene should be cloned into a suitable vector for propagation of the gene.

In a preferred embodiment, DNA can be amplified from genomic or cDNA by polymerase chain reaction (PCR) amplification using primers designed from the known sequence of a related or homologous ND-associated antigen. PCR is used to amplify the desired sequence in DNA clone or a genomic or cDNA library, prior to selection. PCR can be carried out, *e.g.*, by use of a thermal cycler and Taq polymerase (Gene Amp®). The polymerase chain reaction (PCR) is commonly used for obtaining genes or gene fragments of interest. For example, a nucleotide sequence encoding an ND-associated antigen of any desired length can be generated using PCR primers that flank the nucleotide sequence encoding the peptide-binding domain. Alternatively, an ND-associated antigen gene sequence can be cleaved at appropriate sites with restriction endonuclease(s) if such sites are available, releasing a fragment of DNA encoding the ND-associated antigen gene, or an antigenic derivative thereof. If convenient restriction sites are not available, they may be created in the appropriate positions by site-directed mutagenesis and/or DNA amplification methods known in the art (see, for example, Shankarappa *et al.*, 1992, PCR Method Appl. 1: 277-278). The DNA fragment that encodes the ND-associated antigen is then isolated, and ligated into an appropriate expression vector, care being taken to ensure that the proper translation reading frame is maintained.

In an alternative embodiment, for the molecular cloning of an ND-associated antigen gene from genomic DNA, DNA fragments are generated to form a genomic library. Since some of the sequences encoding related ND-associated antigens are available and can be purified and labeled, the cloned DNA fragments in the genomic DNA library may be screened by nucleic acid hybridization to a labeled probe (Benton and Davis, 1977, Science 196: 180; Grunstein and Hogness, 1975, Proc. Natl. Acad. Sci. U.S.A. 72: 3961). Those DNA fragments with substantial homology to the probe will hybridize. It is also possible to

identify an appropriate fragment by restriction enzyme digestion(s) and comparison of fragment sizes with those expected according to a known restriction map.

Alternatives to isolating the ND-associated antigen genomic DNA include, but are not limited to, chemically synthesizing the gene sequence itself from a known sequence or synthesizing a cDNA to the mRNA which encodes the ND-associated antigen. For example, RNA for cDNA cloning of the ND-associated antigen gene can be isolated from cells which express the ND-associated antigen. A cDNA library may be generated by methods known in the art and screened by methods, such as those disclosed for screening a genomic DNA library. If an antibody to the ND-associated antigen is available, the ND-associated antigen may be identified by binding of a labeled antibody to the ND-associated antigen synthesizing clones.

Other specific embodiments for the cloning of a nucleotide sequence encoding an ND-associated antigen, are presented as examples but not by way of limitation, as follows: In a specific embodiment, nucleotide sequences encoding an ND-associated antigen protein can be identified and obtained by hybridization with a probe comprising a nucleotide sequence encoding ND-associated antigen under conditions of low to medium stringency. By way of example and not limitation, procedures using such conditions of low stringency are as follows (see also Shilo and Weinberg, 1981, Proc. Natl. Acad. Sci. U.S.A. 78: 6789-6792). Filters containing DNA are pretreated for 6 h at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 µg/ml denatured salmon sperm DNA. Hybridizations are carried out in the same solution with the following modifications: 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 µg/ml salmon sperm DNA, 10% (wt/vol) dextran sulfate, and 5-20 X 10^6 cpm ^{32}P-labeled probe is used. Filters are incubated in hybridization mixture for 18-20 h at 40°C, and then washed for 1.5 h at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 0.1% SDS. The wash solution is replaced with fresh solution and incubated an additional 1.5 h at 60°C. Filters are blotted dry and exposed for autoradiography. If necessary, filters are washed for a third time at 65-68°C and reexposed to film. Other conditions of low stringency which may be used are well known in the art (*e.g.*, as employed for cross-species hybridizations).

Any technique for mutagenesis known in the art can be used to modify individual nucleotides in a DNA sequence, for purpose of making amino acid substitution(s) in the expressed peptide sequence, or for creating/deleting restriction sites to facilitate further manipulations. Such techniques include but are not limited to, chemical mutagenesis, *in vitro* site-directed mutagenesis (Hutchinson *et al.*, 1978, J. Biol. Chem. 253: 6551), oligonucleotide-directed mutagenesis (Smith, 1985, Ann. Rev. Genet. 19: 423-463; Hill *et al.*, 1987, Methods Enzymol. 155: 558-568), PCR-based overlap extension (Ho

et al., 1989, Gene 77: 51-59), PCR-based megaprimer mutagenesis (Sarkar *et al.*, 1990, Biotechniques 8: 404-407), *etc.* Modifications can be confirmed by double stranded dideoxynucleotide DNA sequencing.

4.1.4.1 Host–Vector Systems

Nucleotide sequences encoding an ND-associated antigenic polypeptide can be inserted into the expression vector for propagation and expression in recombinant cells. An expression construct, as used herein, refers to a nucleotide sequence encoding an ND-associated antigenic polypeptide operably associated with one or more regulatory regions which allows expression of the ND-associated antigenic polypeptide in an appropriate host cell. "Operably-associated" refers to an association in which the regulatory regions and the ND-associated antigenic polypeptide sequence to be expressed are joined and positioned in such a way as to permit transcription, and ultimately, translation of the ND-associated peptide or polypeptide sequence. A variety of expression vectors may be used for the expression of ND-associated antigenic polypeptides, including, but not limited to, plasmids, cosmids, phage, phagemids, or modified viruses. Examples include bacteriophages such as lambda derivatives, or plasmids such as pBR322 or pUC plasmid derivatives or the Bluescript vector (Stratagene). Typically, such expression vectors comprise a functional origin of replication for propagation of the vector in an appropriate host cell, one or more restriction endonuclease sites for insertion of the ND-associated antigenic peptide gene sequence, and one or more selection markers.

Vectors based on *E. coli* are the most popular and versatile systems for high level expression of foreign proteins (Makrides, 1996, Microbiol. Rev. 60: 512-538). Non-limiting examples of prokaryotic expression vectors may include the λgt vector series such as λgt11 (Huynh *et al.*, 1984 in "DNA Cloning Techniques", Vol. I: A Practical Approach (D. Glover, ed.), pp. 49-78, IRL Press, Oxford), and the pET vector series (Studier *et al.*, 1990, Methods Enzymol., 185: 60-89). Non-limiting examples of regulatory regions that can be used for expression in *E. coli* may include but not limited to *lac*, *trp*, *lpp*, *phoA*, *recA*, *tac*, λP_L, and phage T3 and T7 promoters (Makrides, 1996, *supra*).

However, a potential drawback of a prokaryotic host-vector system is the inability to perform many of the post-translational processing events of mammalian cells. Thus, a eukaryotic host-vector system is preferred, a mammalian host-vector system is more preferred, and a human host-vector system is the most preferred. The regulatory regions necessary for transcription of the ND-associated antigenic peptide or polypeptide can be provided by the expression vector. A translation initiation codon (ATG) may also be provided to express a nucleotide sequence encoding an ND-associated antigenic polypeptide that lacks an initiation codon. In a compatible host-construct system, cellular proteins

required for transcription, such as RNA polymerase and transcription factors, will bind to the regulatory regions on the expression construct to effect transcription of the ND-associated antigenic polypeptide sequence in the host organism. The precise nature of the regulatory regions needed for gene expression may vary from host cell to host cell. Generally, a promoter is required which is capable of binding RNA polymerase to initiate the transcription of an operably-associated nucleic acid sequence. Such regulatory regions may include those 5'-non-coding sequences involved with initiation of transcription and translation, such as a TATA box, cap site, a CAAT box, transcription factor binding sites, enhancer elements, and the like. The non-coding region 3' to the coding sequence may contain transcriptional termination regulatory sequences, such as terminators and polyadenylation sites.

Both constitutive and inducible regulatory regions may be used for expression of the ND-associated antigenic peptide or polypeptide. It may be desirable to use inducible promoters when the conditions optimal for growth of the recombinant cells and the conditions for high level expression of the ND-associated antigenic polypeptide are different. Examples of useful regulatory regions are provided in the next section below.

For expression of ND-associated antigenic polypeptides in mammalian host cells, a variety of regulatory regions can be used, for example, the SV40 early and late promoters, the cytomegalovirus (CMV) immediate early promoter, and the Rous sarcoma virus long terminal repeat (RSV-LTR) promoter. Inducible promoters that may be useful in mammalian cells include but are not limited to those associated with the metallothionein II gene, mouse mammary tumor virus glucocorticoid responsive long terminal repeats (MMTV-LTR), the β-interferon gene, and the Hsp70 gene (Williams *et al.*, 1989, Cancer Res. 49: 2735-42 ; Taylor *et al.*, 1990, Mol. Cell. Biol. 10: 165-75).

The following animal regulatory regions, which exhibit tissue specificity and have been utilized in transgenic animals, can also be used in cells of a particular tissue type of interest: elastase I gene control region which is active in pancreatic acinar cells (Swift *et al.*, 1984, Cell 38: 639-646; Ornitz *et al.*, 1986, Cold Spring Harbor Symp. Quant. Biol. 50: 399-409; MacDonald, 1987, Hepatology 7: 425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315: 115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl *et al.*, 1984, Cell 38: 647-658; Adames *et al.*, 1985, Nature 318: 533-538; Alexander *et al.*, 1987, Mol. Cell. Biol. 7: 1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder *et al.*, 1986, Cell 45: 485-495), albumin gene control region which is active in liver (Pinkert *et al.*, 1987, Genes Dev. 1: 268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf *et al.*, 1985, Mol. Cell. Biol. 5: 1639-1648; Hammer *et al.*, 1987, Science 235: 53-58; alpha 1-antitrypsin gene control region which is

active in the liver (Kelsey *et al.*, 1987, Genes Dev. 1: 161-171), beta-globin gene control region which is active in myeloid cells (Mogram *et al.*, 1985, Nature 315: 338-340; Kollias *et al.*, 1986, Cell 46: 89-94; myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead *et al.*, 1987, Cell 48: 703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314: 283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason *et al.*, 1986, Science 234: 1372-1378).

The efficiency of expression of the ND-associated antigenic peptide or polypeptide in a host cell may be enhanced by the inclusion of appropriate transcription enhancer elements in the expression vector, such as those found in SV40 virus, Hepatitis B virus, cytomegalovirus, immunoglobulin genes, metallothionein, β-actin (see Bittner *et al.*, 1987, Methods in Enzymol. 153: 516-544; Gorman, 1990, Curr. Op. in Biotechnol. 1: 36-47).

The expression vector may also contain sequences that permit maintenance and replication of the vector in more than one type of host cell, or integration of the vector into the host chromosome. Such sequences may include but are not limited to replication origins, autonomously replicating sequences (ARS), centromere DNA, and telomere DNA. It may also be advantageous to use shuttle vectors that can be replicated and maintained in at least two types of host cells.

In addition, the expression vector may contain selectable or screenable marker genes for initially isolating or identifying host cells that contain DNA encoding an ND-associated antigenic peptide. For long term, high yield production of ND-associated antigenic peptides, stable expression in mammalian cells is preferred. A number of selection systems may be used for mammalian cells, including, but not limited, to the Herpes simplex virus thymidine kinase (Wigler *et al.*, 1977, Cell 11: 223), hypoxanthine-guanine phosphoribosyltransferase (Szybalski and Szybalski, 1962, Proc. Natl. Acad. Sci. U.S.A. 48: 2026), and adenine phosphoribosyltransferase (Lowy *et al.*, 1980, Cell 22: 817) genes can be employed in *tk*$^-$, *hgprt*$^-$ or *aprt*$^-$ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dihydrofolate reductase (*dhfr*), which confers resistance to methotrexate (Wigler *et al.*, 1980, Natl. Acad. Sci. U.S.A. 77: 3567; O'Hare *et al.*, 1981, Proc. Natl. Acad. Sci. U.S.A. 78: 1527); gpt, which confers resistance to mycophenolic acid (Mulligan and Berg, 1981, Proc. Natl. Acad. Sci. U.S.A. 78: 2072); neomycin phosphotransferase (neo), which confers resistance to the aminoglycoside G-418 (Colberre-Garapin *et al.*, 1981, J. Mol. Biol. 150: 1); and hygromycin phosphotransferase (hyg), which confers resistance to hygromycin (Santerre *et al.*, 1984, Gene 30: 147). Other selectable markers, such as but not limited to histidinol and Zeocin™ can also be used.

In order to insert the DNA sequence of the ND-associated antigenic polypeptide into the cloning site of a vector, DNA sequences with regulatory functions, such as promoters, must be attached to DNA sequences encoding the ND-associated polypeptide. To do this, linkers or adapters providing the appropriate compatible restriction sites may be ligated to the ends of cDNA or synthetic DNA encoding an ND-associated antigenic polypeptide, by techniques well known in the art (Wu *et al.*, 1987, Methods Enzymol. 152: 343-349). Cleavage with a restriction enzyme can be followed by modification to create blunt ends by digesting back or filling in single-stranded DNA termini before ligation. Alternatively, a desired restriction enzyme site can be introduced into a fragment of DNA by amplification of the DNA by use of PCR with primers containing the desired restriction enzyme site.

The expression construct comprising an ND-associated antigenic polypeptide sequence operably associated with regulatory regions can be directly introduced into appropriate host cells for expression and production of ND-associated antigenic peptide-hsp complexes without further cloning (see, for example, U.S. Patent No. 5,580,859). The expression constructs may also contain DNA sequences that facilitate integration of the ND-associated antigenic polypeptide sequence into the genome of the host cell, *e.g.*, via homologous recombination. In this instance, it is not necessary to employ an expression vector comprising a replication origin suitable for appropriate host cells in order to propagate and express the ND-associated antigenic peptide in the host cells.

Expression constructs containing cloned nucleotide sequence encoding ND-associated antigenic polypeptides can be introduced into the host cell by a variety of techniques known in the art, including but not limited to, for prokaryotic cells, bacterial transformation (Hanahan, 1985, in DNA Cloning, A Practical Approach, 1: 109-136), and for eukaryotic cells, calcium phosphate mediated transfection (Wigler *et al.*, 1977, Cell 11: 223-232), liposome-mediated transfection (Schaefer-Ridder *et al.*, 1982, Science 215: 166-168), electroporation (Wolff *et al.*, 1987, Proc. Natl. Acad. Sci. 84: 3344), and microinjection (Cappechi, 1980, Cell 22: 479-488). Co-expression of an ND-associated antigenic peptide and an hsp in the same host cell can be achieved by essentially the same methods.

For long term, high yield production of properly processed ND-associated antigenic polypeptides or ND-associated antigenic peptide–hsp complexes, stable expression in mammalian cells is preferred. Cell lines that stably express ND-associated antigenic polypeptides or ND-associated antigenic peptide–hsp complexes may be engineered by using a vector that contains a selectable marker. By way of example but not limitation, following the introduction of the expression constructs, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective

media. The selectable marker in the expression construct confers resistance to the selection and optimally allows cells to stably integrate the expression construct into their chromosomes and to grow in culture and to be expanded into cell lines. Such cells can be cultured for a long period of time while ND-associated antigenic polypeptide is expressed continuously.

Any of the cloning and expression vectors described herein may be synthesized and assembled from known DNA sequences by techniques well known in the art. The regulatory regions and enhancer elements can be of a variety of origins, both natural and synthetic. Some vectors and host cells may be obtained commercially. Non-limiting examples of useful vectors are described in Appendix 5 of Current Protocols in Molecular Biology, 1988, ed. Ausubel *et al.*, Greene Publish. Assoc. & Wiley Interscience, which is incorporated herein by reference; and the catalogs of commercial suppliers such as Clontech Laboratories, Stratagene Inc., and Invitrogen, Inc.

Alternatively, a number of viral-based expression systems may also be utilized with mammalian cells to produce ND-associated antigens. Vectors using DNA virus backbones have been derived from simian virus 40 (SV40) (Hamer *et al.*, 1979, Cell 17: 725), adenovirus (Van Doren *et al.*, 1984, Mol. Cell Biol. 4: 1653), adeno-associated virus (McLaughlin *et al.*, 1988, J. Virol. 62: 1963), and bovine papillomas virus (Zinn *et al.*, 1982, Proc. Natl. Acad. Sci. 79: 4897). In cases where an adenovirus is used as an expression vector, the donor DNA sequence may be ligated to an adenovirus transcription/translation control region, *e.g.*, the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (*e.g.*, region E1 or E3) will result in a recombinant virus that is viable and capable of expressing heterologous products in infected hosts (see, *e.g.*, Logan and Shenk, 1984, Proc. Natl. Acad. Sci. U.S.A. 81: 3655-3659).

Bovine papillomavirus (BPV) can infect many higher vertebrates, including man, and its DNA replicates as an episome. A number of shuttle vectors have been developed for recombinant gene expression which exist as stable, multicopy (20-300 copies/cell) extrachromosomal elements in mammalian cells. Typically, these vectors contain a segment of BPV DNA (the entire genome or a 69% transforming fragment), a promoter with a broad host range, a polyadenylation signal, splice signals, a selectable marker, and "poisonless" plasmid sequences that allow the vector to be propagated in *E. coli*. Following construction and amplification in bacteria, the expression gene construct is transfected into cultured mammalian cells, for example, by the techniques of calcium phosphate coprecipitation or electroporation. For those host cells that do not manifest a transformed phenotype, selection of transformants is achieved by use of a dominant

selectable marker, such as histidinol and G418 resistance. For example, BPV vectors such as pBCMGSNeo and pBCMGHis may be used to express ND-associated antigenic peptide sequences (Karasuyama *et al.*, Eur. J. Immunol. 18: 97-104; Ohe *et al.*, Human Gene Therapy 6: 325-33) which may then be transfected into a diverse range of cell types for expression of the ND-associated antigenic peptide.

Alternatively, the vaccinia 7.5K promoter may be used (see, *e.g.*, Mackett *et al.*, 1982, Proc. Natl. Acad. Sci. U.S.A. 79: 7415-7419; Mackett *et al.*, 1984, J. Virol. 49: 857-864; Panicali *et al.*, 1982, Proc. Natl. Acad. Sci. U.S.A. 79: 4927-4931) In cases where a human host cell is used, vectors based on the Epstein-Barr virus (EBV) origin (OriP) and EBV nuclear antigen 1 (EBNA-1; a trans-acting replication factor) may be used. Such vectors can be used with a broad range of human host cells, *e.g.*, EBO-pCD (Spickofsky *et al.*, 1990, DNA Prot. Eng. Tech. 2: 14-18), pDR2 and λDR2 (available from Clontech Laboratories).

ND-associated antigenic peptides may also be made with a retrovirus-based expression system. In contrast to transfection, retroviruses can efficiently infect and transfer genes to a wide range of cell types including, for example, primary hematopoietic cells. In retroviruses such as Moloney murine leukemia virus, most of the viral gene sequences can be removed and replaced with nucleic acid sequences encoding the ND-associated antigen, while the missing viral functions can be supplied in *trans*. The host range for infection by a retroviral vector can also be manipulated by the choice of envelope used for vector packaging.

For example, a retroviral vector can comprise a 5' long terminal repeat (LTR), a 3' LTR, a packaging signal, a bacterial origin of replication, and a selectable marker. The ND-associated antigenic peptide DNA is inserted into a position between the 5' LTR and 3' LTR, such that transcription from the 5' LTR promoter transcribes the cloned DNA. The 5' LTR comprises a promoter, including but not limited to an LTR promoter, an R region, a U5 region and a primer binding site, in that order. Nucleotide sequences of these LTR elements are well known in the art. A heterologous promoter as well as multiple drug selection markers may also be included in the expression vector to facilitate selection of infected cells (see McLauchlin *et al.*, 1990, Prog. Nucleic Acid Res. and Molec. Biol. 38: 91-135; Morgenstern *et al.*, 1990, Nucleic Acid Res. 18: 3587-3596; Choulika *et al.*, 1996, J. Virol 70: 1792-1798; Boesen *et al.*, 1994, Biotherapy 6: 291-302; Salmons and Gunzberg, 1993, Human Gene Therapy 4: 129-141; and Grossman and Wilson, 1993, Curr. Opin. in Genetics and Devel. 3: 110-114).

Other useful eukaryotic host-vector system may include yeast and insect systems. In yeast, a number of vectors containing constitutive or inducible promoters may be used with *Saccharomyces cerevisiae* (baker's yeast), *Schizosaccharomyces pombe*

(fission yeast), *Pichia pastoris,* and *Hansenula polymorpha* (methylotropic yeasts). For a review see, Current Protocols in Molecular Biology, Vol. 2, 1988, Ed. S *et al.*, Greene Publish. Assoc. & Wiley Interscience, Ch. 13; Grant *et al.*, 1987, Expression and Secretion Vectors for Yeast, in Methods in Enzymology, Eds. Wu & Grossman, 1987, Acad. Press, N.Y., Vol. 153, pp. 516-544; Glover, 1986, DNA Cloning, Vol. II, IRL Press, Wash., D.C., Ch. 3; and Bitter, 1987, Heterologous Gene Expression in Yeast, Methods in Enzymology, Eds. Berger & Kimmel, Acad. Press, N.Y., Vol. 152, pp. 673-684; and The Molecular Biology of the Yeast *Saccharomyces*, 1982, Eds. Strathern *et al.*, Cold Spring Harbor Press, Vols. I and II.

In an insect system a baculovirus, *Autographa californica* nuclear polyhidrosis virus (AcNPV), can be used as a vector to express an ND-associated antigenic peptide in *Spodoptera frugiperda* cells. The ND-associated antigenic peptide DNA may be cloned into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). These recombinant viruses are then used to infect host cells in which the inserted DNA is expressed (see, *e.g.*, Smith *et al.*, 1983, J. Virol. 46: 584; Smith, U.S. Patent No. 4,215,051).

The expression vector must be used with a compatible host cell which may be derived from a prokaryotic or an eukaryotic organism, including, but not limited to bacteria, yeasts, insects, mammals, and humans. Any cell type that can produce ND-associated antigenic polypeptides and is compatible with the expression vector may be used, including those that have been cultured *in vitro* or genetically engineered. Host cells may be obtained from normal or affected subjects, including healthy humans, patients with neurodegenerative disorders or disease, private laboratory deposits, public culture collections such as the American Type Culture Collection, or from commercial suppliers.

Preferred mammalian host cells include but are not limited to those derived from humans, monkeys and rodents, (see, for example, Kriegler M. in "Gene Transfer and Expression: A Laboratory Manual", New York, Freeman & Co. 1990), such as monkey kidney cell line transformed by SV40 (COS-7, ATCC CRL 1651), human embryonic kidney line (293, 293-EBNA), or 293 cells subcloned for growth in suspension culture (Graham *et al.*, 1977, J. Gen. Virol. 36: 59), baby hamster kidney cells (BHK, ATCC CCL 10), chinese hamster ovary-cells-DHFR (CHO, Urlaub and Chasin. Proc. Natl. Acad. Sci. 77; 4216, 1980), mouse sertoli cells (Mather, 1980, Biol. Reprod. 23: 243-251), mouse fibroblast cells (NIH-3T3), monkey kidney cells (CVI ATCC CCL 70), african green monkey kidney cells (VERO-76, ATCC CRL-1587), human cervical carcinoma cells (HELA, ATCC CCL 2), canine kidney cells (MDCK, ATCC CCL 34), buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75), human liver

cells (Hep G2, HB 8065), and mouse mammary tumor cells (MMT 060562, ATCC CCL51). Exemplary cancer cell types used for demonstrating the utility of recombinant cells (producing ND-associated antigenic peptide-peptide complexes) as a cancer vaccine are provided as follows: mouse fibroblast cell line, NIH3T3, mouse Lewis lung carcinoma cell line, LLC, mouse mastocytoma cell line, P815, mouse lymphoma cell line, EL4 and its ovalbumin transfectant, E.G7, mouse melanoma cell line, B16F10, mouse fibrosarcoma cell line, MC57, and human small cell lung carcinoma cell lines, SCLC#2 and SCLC#7.

The recombinant cells may be cultured under standard conditions of temperature, incubation time, optical density, and media composition. Alternatively, a cells may be cultured under conditions emulating the nutritional and physiological requirements of a cell in which the ND-associated antigen is endogenously expressed.

The ND-associated antigenic protein, or an antigenic portion thereof, can be purified by any methods appropriate for the protein, and then used to form complexes with hsps *in vivo* or *in vitro* as described in Section 4.3, below. Alternatively, as described below, such recombinant cells may be used to co-express ND-associated antigens together with hsps for formation of hsp–ND-associated antigen complexes *in vivo*. However, conditions for growth of recombinant cells may be different from those for expression of ND-associated antigenic polypeptides and hsps. Modified culture conditions and media may be used to enhance production of hsp–peptide complexes. For example, recombinant cells containing ND-associated antigenic polypeptides with their cognate promoters may be exposed to heat or other environmental stress, or chemical stress. Any technique known in the art may be applied to establish the optimal conditions for producing ND-associated antigenic polypeptide or hsp–peptide complexes.

4.1.4.2 Purification Methods for Recombinant ND-Associated Antigens

Generally, the recombinant ND-associated antigenic peptides and polypeptides of the invention can be recovered and purified from recombinant cell cultures by known methods, including ammonium sulfate precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, immunoaffinity chromatography, hydroxyapatite chromatography, and lectin chromatography.

In one embodiment, the invention provides methods for purification of recombinant ND-associated antigenic peptides and polypeptides by affinity purification. The principle of affinity chromatography well known in the art. One approach is based on specific molecular interactions between an affinity label present on the ND-associated antigenic polypeptide and its binding partner. A second approach, immunoaffinity chromatography, relies on the immunospecific binding of an antibody to an epitope present on the tag.

Described below are several methods based on specific molecular interactions of a tag and its binding partner. Protein A affinity chromatography, a method that is generally applicable to purifying recombinant ND-associated antigens that are fused to the constant regions of immunoglobulin, is a well known technique in the art. Staphylococcus protein A is a 42 kD polypeptide that binds specifically to a region located between the second and third constant regions of heavy chain immunoglobulins. Because of the Fc domains of different classes, subclasses and species of immunoglobulins, affinity of protein A for human Fc regions is strong, but may vary with other species. Subclasses that are less preferred include human IgG-3, and most rat subclasses. For certain subclasses, protein G (of Streptococci) may be used in place of protein A in the purification. Protein-A sepharose (Pharmacia or Biorad) is a commonly used solid phase for affinity purification of antibodies, and can be used essentially in the same manner for the purification of ND-associated antigenic polypeptide fused to an immunoglobulin Fc fragment. ND-associated antigenic polypeptide present in cell lysate or, if secreted by the cell, in the supernatant, binds specifically to protein A on the solid phase, while the contaminants are washed away. Bound ND-associated antigenic polypeptide can be eluted by various buffer systems known in the art, including a succession of citrate, acetate and glycine-HCl buffers which gradually lowers the pH. This method is less preferred if the recombinant cells also produce antibodies which will be copurified with the ND-associated antigenic polypeptide. See, for example, Langone, 1982, J. Immunol. Meth. 51: 3; Wilchek *et al.*, 1982, Biochem. Intl. 4: 629; Sjobring *et al.*, 1991, J. Biol. Chem. 26: 399; page 617-618, in Antibodies A Laboratory Manual, edited by Harlow and Lane, Cold Spring Harbor laboratory, 1988.

Alternatively, a polyhistidine tag may be used, in which case, the ND-associated antigenic polypeptide can be purified by metal chelate chromatography. The polyhistidine tag, usually a sequence of six histidines, has a high affinity for divalent metal ions, such as nickel ions (Ni^{2+}), which can be immobilized on a solid phase, such as nitrilotriacetic acid matrices. Polyhistidine has a well characterized affinity for Ni^{2+}-NTA-agarose, and can be eluted with either of two mild treatments: imidazole (0.1-0.2 M) will effectively compete with the resin for binding sites; or lowering the pH just below 6.0 will protonate the histidine side-chains and disrupt the binding. The purification method comprises loading the cell culture supernatant onto the Ni^{2+}-NTA-agarose column, washing the contaminants through, and eluting the ND-associated antigenic polypeptide with imidazole or weak acid. Ni^{2+}-NTA-agarose can be obtained from commercial suppliers such as Sigma (St. Louis) and Qiagen. Antibodies that recognize the polyhistidine tag are also available which can be used to detect and quantify the ND-associated antigenic polypeptide.

Another exemplary affinity label that can be used is the glutathione-S-transferase (GST) sequence, originally cloned from the helminth, *Schistosoma japonicum*. In general, an ND-associated antigenic polypeptide–GST fusion expressed in a prokaryotic host cell, such as *E. coli*, can be purified from the cell culture supernatant by absorption with glutathione agarose beads, followed by elution in the presence of free reduced glutathione at neutral pH. Denaturing conditions are not required at any stage during purification, and therefore, it may be desirable co-purification of hsps and ND-associated polypeptides, for use in the loading of immobilized hsps with ND-associated antigenic peptides. Moreover, since GST is known to form dimers under certain conditions, dimeric ND-associated antigenic peptides may be obtained. See, Smith, 1993, Methods Mol. Cell Bio. 4: 220-229.

Another useful affinity label that can be used is the maltose binding protein (MBP) of *E. coli*, which is encoded by the *mal*E gene. The secreted ND-associated polypeptide-MBP present in the cell supernatant binds to amylose resin while contaminants are washed away. The bound ND-associated polypeptide-MBP is eluted from the amylose resin by maltose. See, for example, Guan *et al.*, 1987, Gene 67: 21-30.

The second approach for purifying ND-associated antigenic peptides is applicable to affinity labels that contain an epitope for which polyclonal or monoclonal antibodies are available. Various methods known in the art for purification of protein by immunospecific binding, such as immunoaffinity chromatography, and immunoprecipitation, can be used. See, for example, Chapter 13 in Antibodies A Laboratory Manual, edited by Harlow and Lane, Cold Spring Harbor laboratory, 1988; and Chapter 8, Sections I and II, in Current Protocols in Immunology, ed. by Coligan *et al.*, John Wiley, 1991; the disclosure of which are both incorporated by reference herein.

The embodiments described above may also be used to recover and purify hsp –ND-associated antigenic peptide complexes from the cell culture medium of mammalian cells, such as human cells expressing an hsp-ND-associated peptide complex of the invention. The methods can be adapted to perform medium and large scale purification of an ND-associated antigenic peptide and/or hsp –ND-associated antigenic peptide complexes. Methods that do not require lowering pH or denaturing conditions are most preferred for purification of hsp-antigenic peptide complexes. The methods may be used to isolate ND-associated antigenic peptides from eukaryotic cells, for example, cells of neuronal origin or cells isolated from a subject with a neurodegenerative disorder.

4.2 Sources of Heat Shock Proteins

Amino acid sequences and nucleotide sequences of many hsps are generally available in sequence databases, such as GenBank. Computer programs, such as Entrez, can be used to browse the database, and retrieve any amino acid sequence and genetic sequence data of interest by accession number. These databases can also be searched to identify sequences with various degrees of similarities to a query sequence using programs, such as FASTA and BLAST, which rank the similar sequences by alignment scores and statistics. Such nucleotide sequences of non-limiting examples of hsps that can be used for the compositions, methods, and for preparation of the hsp peptide-complexs of the invention are as follows: human hsp70, Genbank Accession No. M24743, Hunt *et al.*, 1995, Proc. Natl. Acad. Sci. U.S.A., 82: 6455-6489; human Hsp90, Genbank Accession No. X15183, Yamazaki *et al.*, Nucl. Acids Res. 17: 7108; human gp96: Genbank Accession No. X15187, Maki *et al.*, 1990, Proc. Natl. Acad. Sci. U.S.A. 87: 5658-5562; human BiP: Genbank Accession No. M19645; Ting *et al.*, 1988, DNA 7: 275-286; human Hsp27, Genbank Accession No. M24743; Hickey *et al.*, 1986, Nucleic Acids Res. 14: 4127-45; mouse Hsp70: Genbank Accession No. M35021, Hunt *et al.*, 1990, Gene 87: 199-204; mouse gp96: Genbank Accession No. M16370, Srivastava *et al.*, 1987, Proc. Natl. Acad. Sci. U.S.A. 85: 3807-3811; and mouse BiP: Genbank Accession No. U16277, Haas *et al.*, 1988, Proc. Natl. Acad. Sci. U.S.A. 85: 2250-2254. Due to the degeneracy of the genetic code, the term "hsp gene", as used herein, refers not only to the naturally occurring nucleotide sequence but also encompasses all the other degenerate DNA sequences that encode the hsp.

Once the nucleotide sequence of an hsp has been identified, hsps can be produced by any method known in the art, such as the recombinant methods described above (Section 4.1.3) for the production of ND-associated antigens. hsp-encoding cDNA or genomic DNA may be obtained from any species, for example, by PCR amplification. Oligonucleotide primers representing known nucleic acid sequences of related hsps can be used as PCR primers. In a preferred aspect, the oligonucleotide primers represent at least part of the hsp gene that is highly conserved between hsps of different species. One can choose to synthesize several different degenerate primers for use in the PCR reactions. It is also possible to vary the stringency of hybridization conditions used in priming the PCR reactions, to allow for greater or lesser degrees of nucleotide sequence similarity between the known hsp nucleotide sequence and the nucleic acid homolog being isolated. For cross species hybridization, low stringency conditions are preferred. For same species hybridization, moderately stringent conditions are preferred. After successful amplification, the sequence encoding an hsp may be cloned and sequenced. If the size of the coding region of the hsp gene being amplified is too large to be amplified in a single PCR, several

PCR covering the entire gene, preferably with overlapping regions, may be carried out, and the products of the PCR ligated together to form the entire coding sequence. Alternatively, if a segment of an hsp gene is amplified, that segment may be cloned, and utilized as a probe to isolate a complete cDNA or genomic clone. The hsp gene may then be cloned into an appropriate expression vector, and be produced, propogated, isolated and purified according to methods for recombinant production of proteins, such as those described for recombinant production of ND-associated antigens described in Section 4.1.2, above.

An alternative to producing hsps by recombinant techniques is peptide synthesis. For example, an hsp, or a fragment thereof, can be synthesized using methods known in the art, such as those described for the synthesis of ND-associated antigens, in Section 4.1.2, above. In this case, conventional peptide synthesis may be used, or other synthetic protocols well known in the art.

Purification of the resulting hsp, or fragment thereof, may then be accomplished using conventional procedures, such as preparative HPLC using gel permeation, partition and/or ion exchange chromatography. The choice of appropriate matrices and buffers are well known in the art.

4.3 Complexes of Hsps and ND-Associated Antigenic Peptides

Complexes of hsps associated with peptides can elicit an immune response, by stimulating CD4 helper T lymphocytes and/or CD8 cytotoxic T lymphocytes (CTL) that recognize the hsp-associated peptides and by stimulating antibody production. For example, in the case of cancer-specific antigens, when mice are injected with hsps isolated from tumor cells (donor cells), such mice produce CTL specific for donor cell peptides in association with the responder mouse's class I MHC proteins (Janetzki et al., 1998, J. Immunotherapy 21: 269-276). Thus, antigenic peptides associated with hsps can enter the MHC class I presenting pathway of professional antigen presenting cells (APCs). Such a method can be used to elicit an immune response against ND-associated antigens for the prevention and treatment of neurodegenerative disorders. Described herein are methods for producing such hsp-ND-associated antigen complexes for use in immunotherapy.

4.3.1. Recombinant Expression and Production of ND-Associated Antigen-Hsp Complexes

The purification of Hsp70–peptide complexes from cell lysates has been described previously, see, for example, Udono *et al.*, 1993, J. Exp. Med. 178: 1391-1396. The purification of Hsp90–peptide complexes and gp96–peptide complexes from cell lysates have been described, for example, in WO 95/24923, dated September 21, 1995, and WO 97/10000, dated March 20, 1997. These methods can be used to purify the hsp–ND-

associated antigenic peptide complexes of the invention from the recombinant cells and/or the cell culture.

The invention provides methods for purification of hsp–ND-associated antigenic peptide complexes by affinity purification, based on the properties of the affinity label present on the hsp or the ND-associated antigenic peptide. One approach is based on specific molecular interactions between a tag and its binding partner. The other approach relies on the immunospecific binding of an antibody to an epitope present on the tag. The principle of affinity chromatography well known in the art is generally applicable to both of these approaches.

To produce hsp–ND-associated antigenic peptide complexes, a nucleotide sequence encoding an hsp can be introduced into cells including, but not limited to, epithelial cells, endothelial cells, keratinocytes, fibroblasts, muscle cells, hepatocytes; blood cells such as T lymphocytes, B lymphocytes, monocytes, macrophages, neutrophils, eosinophils, megakaryocytes, granulocytes; various stem or progenitor cells, in particular hematopoietic stem or progenitor cells, *e.g.*, as obtained from bone marrow, umbilical cord blood, peripheral blood, fetal liver, *etc.* The choice of cell type depends on the type of ND-associated antigen being expressed, and can be determined by one of skill in the art. Preferably, the cell chosen produces the ND-associated antigen of interest. In an alternative embodiment, the ND-associated antigen can be engineered to be expressed in a cell type that does not normally express the ND-associated antigen.

In a specific embodiment, an expression construct comprising a nucleic acid sequence encoding the hsp is introduced into an antigenic cell. As used herein, antigenic cells may include cells that are of neural origin, or may include cells transformed with a nucleic acid such that they express an ND-associated antigenic peptide.

Preferably, the cells, *e.g.*, neural cells, used in the methods of the invention are of mammalian origin. Mammals contemplated by this aspect of the invention include humans, companion animals (*e.g.*, dogs and cats), livestock animals (*e.g.*, sheep, cattle, goats, pigs and horses), laboratory animals (*e.g.*, mice, rats and rabbits), and captive or free wild animals.

In various embodiments, any neural cells, preferably human neural cells, can be used in the present methods for producing hsp–peptide complexes, or for use as a vaccine. The cells provide the antigenic peptides which become associated non-covalently with the expressed hsp. Introduction of gene constructs encoding the hsp and ND-associated antigen can be carried out by any method known in the art, including gene therapy art, such as but not limited to transfection, electroporation, microinjection, infection with a viral or bacteriophage vector containing the nucleic acid sequence encoding the hsp and ND-associated antigen, cell fusion, chromosome-mediated gene transfer, microcell-

mediated gene transfer, spheroplast fusion, *etc.* Numerous techniques are known in the art for the introduction of foreign genes into cells (see *e.g.*, Loeffler and Behr, 1993, Meth. Enzymol. 217: 599-618; Cohen *et al.*, 1993, Meth. Enzymol. 217: 618-644; Cline, 1985, Pharmac. Ther. 29: 69-92) may be used in accordance with the present invention, provided that the necessary developmental and physiological functions of the recipient cells are not disrupted. The technique should provide for the stable transfer of the nucleic acid sequence encoding the hsp and ND-associated antigen to the cell, so that the sequence is expressible and preferably heritable and expressible by its cell progeny.

4.3.1.1 Preparation of Hsp70–Peptide Complexes

The purification of hsp70–peptide complexes has been described previously, see, for example, Udono *et al.*, 1993, J. Exp. Med. 178: 1391-1396. The following procedure may be used, presented by way of example but not limitation, to purify hsp70 complexes from cells. Initially, cells are suspended in 3 volumes of 1X Lysis buffer consisting of 5mM sodium phosphate buffer, pH 7, 150mM NaCl, 2mM $CaCl_2$, 2mM $MgCl_2$ and 1mM phenyl methyl sulfonyl fluoride (PMSF). Then, the pellet is sonicated, on ice, until >99% cells are lysed as determined by microscopic examination. As an alternative to sonication, the cells may be lysed by mechanical shearing and in this approach the cells typically are resuspended in 30mM sodium bicarbonate pH 7.5, 1mM PMSF, incubated on ice for 20 minutes and then homogenized in a Dounce homogenizer until >95% cells are lysed.

The lysate is then centrifuged at 1,000g for 10 minutes to remove unbroken cells, nuclei and other cellular debris. The resulting supernatant is recentrifuged at 100,000g for 90 minutes, the supernatant harvested and then mixed with Con A Sepharose equilibrated with phosphate buffered saline (PBS) containing 2mM Ca^{2+} and 2mM Mg^{2+}. When the cells are lysed by mechanical shearing the supernatant is diluted with an equal volume of 2X lysis buffer prior to mixing with Con A Sepharose. The supernatant is then allowed to bind to the Con A Sepharose for 2-3 hours at 4°C. The material that fails to bind is harvested and dialyzed for 36 hours (three times, 100 volumes each time) against 10mM Tris-Acetate pH 7.5, 0.1mM EDTA, 10mM NaCl, 1mM PMSF. Then the dialyzate is centrifuged at 17,000 rpm (Sorvall SS34 rotor) for 20 minutes. Then the resulting supernatant is harvested and applied to a Mono Q FPLC column equilibrated in 20mM Tris-acetate pH 7.5, 2mM NaCl, 0.1mM EDTA and 15mM 2-mercaptuethanol). The column is then developed with a 20mM to 500mM NaCl gradient and then eluted fractions fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and characterized by immunoblotting using an appropriate anti-Hsp70 antibody (such as from clone N27F3-4, from StressGen).

Fractions strongly immunoreactive with the anti-hsp70 antibody are pooled and the Hsp70–peptide complexes precipitated with ammonium sulfate; specifically with a 50%-70% ammonium sulfate cut. The resulting precipitate is then harvested by centrifugation at 17,000 rpm (SS34 Sorvall rotor) and washed with 70% ammonium sulfate. The washed precipitate is then solubilized and any residual ammonium sulfate removed by gel filtration on a Sephadex^R G25 column (Pharmacia). If necessary the hsp70 preparation thus obtained can be repurified through the MonoQ FPLC Column as described above.

The hsp70–peptide complex can be purified to apparent homogeneity using this method. Typically 1 mg of hsp70–peptide complex can be purified from 1 g of cells/tissue.

An alternative method for purification of hsp70–peptide complexes comprises contacting cellular proteins with ADP or a nonhydrolyzable analog of ATP affixed to a solid substrate, such that hsp70 in the lysate can bind to the ADP or nonhydrolyzable ATP analog, and eluting the bound hsp70. A preferred method uses column chromatography with ADP affixed to a solid substratum (*e.g.*, ADP-agarose). The resulting hsp70 preparations are higher in purity and devoid of non-specifically bound peptides. The hsp70 yields are also increased significantly by about more than 10 fold. Alternatively, chromatography with nonhydrolyzable analogs of ATP, instead of ADP, can be used for purification of hsp70–peptide complexes. By way of example, but not limitation, purification of hsp70–peptide complexes by ADP-agarose chromatography can be carried out as follows: cells (500 million cells) are homogenized in hypotonic buffer and the lysate is centrifuged at 100,000 g for 90 minutes at 4°C. The supernatant is applied to an ADP-agarose column. The column is washed in buffer and is eluted with 5 column volumes of 3 mM ADP. The hsp70–peptide complexes elute in fractions 2 through 10 of the total 15 fractions which elute. The eluted fractions are analyzed by SDS-PAGE. The hsp70–peptide complexes can be purified to apparent homogeneity using this procedure.

4.3.1.2 Preparation of Hsp90–Peptide Complexes

The purification of hsp90–peptide complexes from cell lysates has been described, for example, in WO 95/24923, dated September 21, 1995, and WO 97/10000, dated March 20, 1997. A procedure that can be used, presented by way of example and not limitation, is as follows: Initially, neural cells are suspended in 3 volumes of 1X Lysis buffer consisting of 5mM sodium phosphate buffer (pH7), 150mM NaCl, 2mM $CaCl_2$, 2mM $MgCl_2$ and 1mM phenyl methyl sulfonyl fluoride (PMSF). Then, the pellet is sonicated, on ice, until >99% cells are lysed as determined by microscopic examination. As an alternative to sonication, the cells may be lysed by mechanical shearing and in this approach the cells typically are resuspended in 30mM sodium bicarbonate pH 7.5, 1mM

PMSF, incubated on ice for 20 minutes and then homogenized in a Dounce homogenizer until >95% cells are lysed.

Then the lysate is centrifuged at 1,000g for 10 minutes to remove unbroken cells, nuclei and other cellular debris. The resulting supernatant is recentrifuged at 100,000g for 90 minutes, the supernatant harvested and then mixed with Con A Sepharose equilibrated with PBS containing 2mM Ca^{2+} and 2mM Mg^{2+}. When the cells are lysed by mechanical shearing the supernatant is diluted with an equal volume of 2X Lysis buffer prior to mixing with Con A Sepharose. The supernatant is then allowed to bind to the Con A Sepharose for 2-3 hours at 4°C. The material that fails to bind is harvested and dialyzed for 36 hours (three times, 100 volumes each time) against 20mM Sodium phosphate pH 7.4, 1mM EDTA, 250mM NaCl, 1mM PMSF. Then the dialyzate is centrifuged at 17,000 rpm (Sorvall SS34 rotor) for 20 minutes. Then the resulting supernatant is harvested and applied to a Mono Q FPLC column equilibrated with dialysis buffer. The proteins are then eluted with a salt gradient of 200mM to 600mM NaCl.

The eluted fractions are fractionated by SDS-PAGE and fractions containing the hsp90–peptide complexes identified by immunoblotting using an anti-Hsp90 antibody such as 3G3 (Affinity Bioreagents). Hsp90–peptide complexes can be purified to apparent homogeneity using this procedure. Typically, 150-200 μg of hsp90–peptide complex can be purified from 1g of cells/tissue.

4.3.1.3 Preparation of gp96–Peptide Complexes

The purification of gp96–peptide complexes from cell lysates has been described, for example, in WO 95/24923, dated September 21, 1995, and WO 97/10000, dated March 20, 1997. A procedure that can be used, presented by way of example and not limitation, is as follows: A cell pellet is resuspended in 3 volumes of buffer consisting of 30mM sodium bicarbonate buffer (pH 7.5) and 1mM PMSF and the cells allowed to swell on ice 20 minutes. The cell pellet is then homogenized in a Dounce homogenizer (the appropriate clearance of the homogenizer will vary according to each cell type) on ice until >95% cells are lysed.

The lysate is centrifuged at 1,000g for 10 minutes to remove unbroken cells, nuclei and other debris. The supernatant from this centrifugation step is then recentrifuged at 100,000g for 90 minutes. The gp96–peptide complex can be purified either from the 100,000 pellet or from the supernatant.

When purified from the supernatant, the supernatant is diluted with equal volume of 2X lysis buffer and the supernatant mixed for 2-3 hours at 4°C with ConA Sepharose equilibrated with PBS containing 2mM Ca^{2+} and 2mM Mg^{2+}. Then, the slurry is packed into a column and washed with 1X lysis buffer until the OD_{280} drops to baseline.

Then, the column is washed with 1/3 column bed volume of 10% α-methyl mannoside (α-MM) dissolved in PBS containing 2mM Ca^{2+} and 2mM Mg^{2+}, the column sealed with a piece of parafilm, and incubated at 37°C for 15 minutes. Then the column is cooled to room temperature and the parafilm removed from the bottom of the column. Five column volumes of the α-MM buffer are applied to the column and the eluate analyzed by SDS-PAGE. Typically the resulting material is about 60-95% pure, however this depends upon the cell type and the tissue-to-lysis buffer ratio used. Then the sample is applied to a Mono Q FPLC column (Pharmacia) equilibrated with a buffer containing 20mM sodium phosphate, pH 7.4. The proteins are then eluted from the column with a 0-1M NaCl gradient and the gp96 fraction elutes between 400mM and 550mM NaCl.

The procedure, however, may be modified by two additional steps, used either alone or in combination, to consistently produce apparently homogeneous gp96-peptide complexes. One optional step involves an ammonium sulfate precipitation prior to the Con A purification step and the other optional step involves DEAE-Sepharose purification after the Con A purification step but before the Mono Q FPLC step.

In the first optional step, described by way of example as follows, the supernatant resulting from the 100,000g centrifugation step is brought to a final concentration of 50% ammonium sulfate by the addition of ammonium sulfate. The ammonium sulfate is added slowly while gently stirring the solution in a beaker placed in a tray of ice water. The solution is stirred from about 1/2 to 12 hours at 4°C and the resulting solution centrifuged at 6,000 rpm (Sorvall SS34 rotor). The supernatant resulting from this step is removed, brought to 70% ammonium sulfate saturation by the addition of ammonium sulfate solution, and centrifuged at 6,000 rpm (Sorvall SS34 rotor). The resulting pellet from this step is harvested and suspended in PBS containing 70% ammonium sulfate in order to rinse the pellet. This mixture is centrifuged at 6,000 rpm (Sorvall SS34 rotor) and the pellet dissolved in PBS containing 2mM Ca^{2+} and Mg^{2+}. Undissolved material is removed by a brief centrifugation at 15,000 rpm (Sorvall SS34 rotor). Then, the solution is mixed with Con A Sepharose and the procedure followed as before.

In the second optional step, described by way of example as follows, the gp96 containing fractions eluted from the Con A column are pooled and the buffer exchanged for 5mM sodium phosphate buffer, pH 7, 300mM NaCl by dialysis, or preferably by buffer exchange on a Sephadex G25 column. After buffer exchange, the solution is mixed with DEAE-Sepharose previously equilibrated with 5mM sodium phosphate buffer, pH 7, 300mM NaCl. The protein solution and the beads are mixed gently for 1 hour and poured into a column. Then, the column is washed with 5mM sodium phosphate buffer, pH 7, 300mM NaCl, until the absorbance at 280nm drops to baseline.

Then, the bound protein is eluted from the column with five volumes of 5mM sodium phosphate buffer, pH 7, 700mM NaCl. Protein containing fractions are pooled and diluted with 5mM sodium phosphate buffer, pH 7 in order to lower the salt concentration to 175mM. The resulting material then is applied to the Mono Q FPLC column (Pharmacia) equilibrated with 5mM sodium phosphate buffer, pH 7 and the protein that binds to the Mono Q FPLC column (Pharmacia) is eluted as described before.

It is appreciated, however, that one skilled in the art may assess, by routine experimentation, the benefit of incorporating the second optional step into the purification protocol. In addition, it is appreciated also that the benefit of adding each of the optional steps will depend upon the source of the starting material.

When the gp96 fraction is isolated from the 100,000g pellet, the pellet is suspended in 5 volumes of PBS containing either 1% sodium deoxycholate or 1% octyl glucopyranoside (but without the Mg^{2+} and Ca^{2+}) and incubated on ice for 1 hour. The suspension is centrifuged at 20,000g for 30 minutes and the resulting supernatant dialyzed against several changes of PBS (also without the Mg^{2+} and Ca^{2+}) to remove the detergent. The dialysate is centrifuged at 100,000g for 90 minutes, the supernatant harvested, and calcium and magnesium are added to the supernatant to give final concentrations of 2mM, respectively. Then the sample is purified by either the unmodified or the modified method for isolating gp96–peptide complex from the 100,000g supernatant, see above.

The gp96–peptide complexes can be purified to apparent homogeneity using this procedure. About 10-20μg of gp96 can be isolated from 1g cells/tissue.

4.3.2 In Vitro Complexing

In an alternative embodiment, complexes of hsps with ND-associated antigenic molecules are produced *in vitro*. Immunogenic hsp–ND-associated peptide complexes can be generated *in vitro* by covalent or non-covalent coupling of an hsp with an ND-associated antigenic peptide. As described in Sections 4.1.2 and 4.1.3 above, antigenic molecules may be isolated from various sources, chemically synthesized, or produced recombinantly. Hsps may also be prepared by a variety of methods, as described in Section 4.2, above. After isolation of hsps and antigenics molecules, complexes are produced *in vitro*. Procedures for forming such hsp–ND-associated peptide complexes and preferred, exemplary protocols for covalent and noncovalently are provided herein. Such methods can be readily adapted for medium or large scale production of the immunotherapeutic or prophylactic vaccines of the invention.

4.3.2.1 Formation of Non-covalent ND-Associated Antigen–hsp Complexes

This section describes a preferred method for preparing non-covalent immunogenic complexes of hsps and ND-associated peptides.

Hsps are prepared, as described in Section 4.2, above. Prior to complexing, the hsps may be pretreated with ATP or low pH to remove any peptides that may be associated with the hsp of interest. It may be advantageous to use hsps that are reversibly bound to a solid phase to facilitate buffer exchange, washings and isolation of the complexes before or after the complexing reaction. When the ATP procedure is used, excess ATP is removed from the preparation by the addition of apyranase as described by Levy *et al.*, 1991, Cell 67: 265-274. When the low pH procedure is used, the buffer is readjusted to neutral pH by the addition of pH modifying reagents.

The ND-associated antigenic molecules (1μg) and the pretreated hsp (9μg) are mixed together to give an approximately 5:1 antigenic molecule : hsp molar ratio. The mixture is then incubated for 15 minutes to 3 hours at 4° to 45°C in a suitable binding buffer such as one containing 20mM sodium phosphate, pH 7.2, 350mM NaCl, 3mM $MgCl_2$ and 1mM phenyl methyl sulfonyl fluoride (PMSF). The preparations are centrifuged through a Centricon 10 assembly (Millipore) to remove any unbound peptide. If the hsp is bound to a solid phase, the hsp-ND-associated peptide complexes formed can be washed free of unbound peptide prior to eluting the hsp-peptide complex off the solid phase. The association of the peptides with the hsp can be assayed by SDS-PAGE.

In an alternative embodiment, preferred for producing complexes of Hsp70 to ND-associated antigenic molecules, 5-10 micrograms of purified hsp is incubated with equimolar quantities of the antigenic molecule in 20mM sodium phosphate buffer pH 7.5, 0.5M NaCl, 3mM $MgCl_2$ and 1mM ADP in a volume of 100 microliter at 37°C for 1 hr. This incubation mixture is further diluted to 1 ml in phosphate-buffered saline.

In another alternative embodiment of the invention, preferred for producing complexes of gp96 to peptides, 5-10 micrograms of gp96 immobilized by its affinity tag to a solid phase is incubated with equimolar or excess quantities of the antigenic peptide in a suitable buffer, such as one containing 20mM sodium phosphate buffer pH 7.5, 0.5M NaCl, 3nM MgCl2 at about 50°C for about 10 minutes. For example, modified gp96 containing the Ig tag can be immobilized to protein A-Sepharose for this procedure. This incubation mixture is then further incubated for about 30 minutes at room temperature. The solid phase with the bound hsp-peptide complexes is washed several times to remove any unbound peptide. The hsp-peptide complexes is then eluted from the solid phase by the appropriate technique.

Following complexing, the immunogenic hsp-ND-associated peptide complexes can optionally be assayed *in vitro* using, for example, the methods described in Section 4.4.3, below, and optionally characterized further in animal models using the preferred administration protocols and excipients discussed in Section 4.6.1 below

4.3.2.2 Formation of Covalent ND-Associated Antigen-Hsp Complexes

As an alternative to non-covalent complexes, ND-associated antigenic peptides covalently attached to hsps may be used as vaccines to elicit an immune response.

To prepare such covalent hsp–ND-associated antigenic peptide complexes, hsps and ND-associated antigenic peptides are prepared, as described in Sections 4.1.2 and 4.1.3, and Section 4.2, respectively. Prior to complexing, the hsps may be pretreated with ATP or low pH to remove any peptides that may be associated with the hsp of interest. Non-covalent hsps and ND-associated antigenic peptide complexes can then be prepared, as described in Section 4.3.2.1, above. Finally, the hsp and the ND-associated peptide within such complexes are covalently coupled.

In one embodiment, hsps may be covalently coupled to ND-associated antigen by chemical crosslinking. Chemical crosslinking methods are well known in the art. For example, in a preferred embodiment, glutaraldehyde crosslinking may be used. Glutaradehyde crosslinking has been used for formation of covalent complexes of peptides and hsps (see Barrios *et al.*, 1992, Eur. J. Immunol. 22: 1365-1372). Preferably, 1 mg of hsp is crosslinked to 1 mg of ND-associated peptide in the presence of 0.002% glutaraldehyde for 2 hours. Glutaraldehyde is removed by dialysis against phosphate buffered saline (PBS) overnight (Lussow *et al.*, 1991, Eur. J. Immunol. 21: 2297-2302). Other methods for chemical crosslinking may also be used, in addition other methods for covalent attachment of proteins, such as photocrosslinking (See Current Protocols in Molecular Biology, Ausubel *et al.* (eds.), Greene Publishing Associates and Wiley Interscience, New York).

4.3.3. ND-Associated Antigen-Hsp Fusion Proteins

In another embodiment, recombinant fusion proteins, comprised of a heat shock protein sequence and an ND-associated antigenic peptide sequence, may be used to treat or prevent neurodegenerative diseases and disorders. To produce such a recombinant fusion protein, an expression vector is constructed using nucleic acid sequences encoding a heat shock protein fused to sequences encoding an ND-associated antigen, using recombinant methods known in the art, such as those described in Section 4.1.2, above. Hsp-antigenic peptide fusions are then expressed and isolated. Such fusion proteins can be used to elicit an immune response (Suzue *et al.*, 1997, Proc. Natl. Acad. Sci. U.S.A. 94:

13146-51). By specifically designing the antigenic peptide portion of the molecule, such fusion proteins can be used to elicit an immune response and in immunotherapy against target neurodegenerative disorders.

4.4 Therapeutic Uses of Complexes of Hsps and ND-Associated Antigenic Molecules

The present invention encompasses the use of hsps in methods for treatment and prevention of neurodegenerative disorders. In various embodiments described in detail herein, an effective amount of an hsp in a complex with antigenic molecule that displays the antigenicity of an antigen associated with a neurodegenerative disorder is administered to a patient for therapeutic purposes.

4.4.1 Prevention and Treatment of Neurodegenerative Disorders

For prevention and treatment of neurodegenerative disorders, hsp-ND-associated antigen complexes are prepared from a cell that displays the antigenicity of an antigen of an neurodegenerative disease or disorder, and used as vaccines against the disease or disorder. As will be appreciated by those skilled in the art, the protocols described herein may be used to isolate hsp–peptide complexes from any cell that displays the antigenicity of an antigen associated with the neurodegenerative disorder. For example, cells may express the ND-associated antigen itself, or alternatively, cells may be infected by or engineered to express a fragment or non-pathogenic form of the ND-associated antigen.

In one embodiment, a method for treatment or prevention of an neuropsychiatric disorder comprises introducing into a cell that displays the antigenicity of an ND-associated antigen an expressible hsp gene sequence, preferably as an expression gene construct. The hsp gene sequence is manipulated by methods described above in Section 4.2.3, so that the hsp gene sequence, in the form of an expression construct, located extrachromosomally or integrated in the chromosome, is suitable for expression of the hsp in the recombinant cells. The recombinant cells containing the expression gene constructs are cultured under conditions such that hsps encoded by the expression gene construct are expressed. Complexes of hsps associated with the antigenic molecule that displays the antigenicity of an antigen associated with a neurodegenerative disorder are purified from the cell culture or culture medium by the methods described in Section 4.3.

In various embodiments, hsp–peptide complexes are prepared from a cell genetically manipulated to express an hsp, for example, tissues, isolated cells or immortalized eukaryotic cell lines transformed with an ND-associated antigen. When immortalized animal cell lines are used as a source of the hsp–peptide complex, it is

important to use cell lines that can express the ND-associated antigen of interest. In addition, it is preferable to use cells that are derived from the same species as the intended recipient of the vaccine. Techniques for introducing an expressible form of the hsp gene sequences into these cell lines are described above in Section 4.2.3.

Neurodegenerative disorders include disorders relating to the central nervous system and/or peripheral nervous system including, but not limited to, cognitive and neurodegenerative disorders such as Alzheimer's Disease, age-related loss of cognitive function and senile dementia, Parkinson's disease, amyotrophic lateral sclerosis, Wilson's Disease, cerebral and progressive supranuclear palsy, Guam disease, Lewy body dementia, prion diseases, such as spongiform encephalopathies, *e.g.*, Creutzfeldt-Jakob disease, polyglutamine diseases, such as Huntington's disease, myotonic dystrophy, Freidrich's ataxia and other ataxias, well as Gilles de la Tourette's syndrome, seizure disorders such as epilepsy and chronic seizure disorder, stroke, brain or spinal cord trauma, AIDS dementia, alcoholism, autism, retinal ischemia, glaucoma, autonomic function disorders such as hypertension and sleep disorders, and neuropsychiatric disorders that include, but are not limited to schizophrenia, schizoaffective disorder, attention deficit disorder, dysthymic disorder, major depressive disorder, mania, obsessive-compulsive disorder, psychoactive substance use disorders, anxiety, panic disorder, as well as unipolar and bipolar affective disorders. Additional neuropsychiatric and neurodegenerative disorders include, for example, those listed in the American Psychiatric Association's Diagnostic and Statistical manual of Mental Disorders (DSM), the most current version of which is incorporated herein by reference in its entirety.

In a preferred aspect of the invention, the purified hsp-ND-associated antigen complex vaccines may have particular utility in the treatment of human neurodegenerative disorders. It is appreciated, however, that the vaccines developed using the principles described herein will be useful in treating diseases of other mammals, for example, farm animals including: cattle; horses; sheep; goats; and pigs, and household pets including: cats; and dogs, that have similar pathologies.

4.4.2 Combination With Adoptive Immunotherapy

Adoptive immunotherapy refers to a therapeutic approach for treating neurodegenerative diseases in which immune cells are administered to a host with the aim that the cells mediate specific immunity, either directly or indirectly, to cells that express ND-associated antigens and/or antigenic components, and result in treatment of the neurodegenerative disorder, or prevention of the neurodegenerative disorder, as the case may be (see U.S. Patent Application Serial No. 08/527,546, filed September 13, 1995, which is incorporated by reference herein in its entirety). The use of hsp-peptide complexes

for sensitizing antigen presenting cells *in vitro* for use in adoptive immunotherapy is described in PCT publication WO 97/10002, dated March 20, 1997, which is incorporated by reference herein in its entirety. Methods for sensitizing antigen presenting cells (APC) using hsps in complexes with antigenic (or immunogenic) molecules, for adoptive immunotherapy are described in detail herein.

According to the invention, therapy by administration of hsp–peptide complexes, using any desired route of administration, is combined with adoptive immunotherapy using APC sensitized with hsp–antigenic molecule complexes. The hsp–peptide complex-sensitized APC can be administered concurrently with hsp–peptide complexes, or before or after administration of hsp– peptide complexes. Furthermore, the mode of administration can be varied, including but not limited to, *e.g.*, subcutaneously, intravenously, intraperitoneally, intramuscularly, intradermally or mucosally.

4.4.2.1 Sensitization of Macrophages and Antigen Presenting Cells with HSP– Peptide Complexes

The antigen-presenting cells, including but not limited to macrophages, dendritic cells and B-cells, are preferably obtained by production *in vitro* from stem and progenitor cells from human peripheral blood or bone marrow as described by Inaba *et al.*, 1992, J. Exp. Med. 176:1693-1702. APC can be obtained by any of various methods known in the art. In a preferred aspect human macrophages are used, obtained from human blood cells.

By way of example, but not limitation, macrophages can be obtained as follows: Mononuclear cells are isolated from peripheral blood of a patient (preferably the patient to be treated), by Ficoll-Hypaque gradient centrifugation and are seeded on tissue culture dishes which are pre-coated with the patient's own serum or with other AB+ human serum. The cells are incubated at 37°C for 1 hr, then non-adherent cells are removed by pipetting. To the adherent cells left in the dish, is added cold (4°C) 1 mM EDTA in phosphate-buffered saline and the dishes are left at room temperature for 15 minutes. The cells are harvested, washed with RPMI buffer and suspended in RPMI buffer. Increased numbers of macrophages may be obtained by incubating at 37°C with macrophage-colony stimulating factor (M-CSF); increased numbers of dendritic cells may be obtained by incubating with granulocyte-macrophage-colony stimulating factor (GM-CSF) as described in detail by Inaba, K., *et al.*, 1992, J. Exp. Med. 176:1693-1702.

APC are sensitized with hsps bound to ND-associated antigenic molecules by incubating the cells *in vitro* with the complexes. The APC are sensitized with complexes of hsps and antigenic molecules preferably by incubating *in vitro* with the hsp–complex at 37°C for 15 minutes to 24 hours. By way of example but not limitation, $4x10^7$

macrophages can be incubated with 10 microgram gp96-peptide complexes per ml or 100 microgram hsp90-peptide complexes per ml at 37°C for 15 minutes-24 hours in 1 ml plain RPMI medium. The cells are washed three times and resuspended in a physiological medium preferably sterile, at a convenient concentration (*e.g.*, $1x10^7$/ml) for injection in a patient. In a preferred embodiment, the antigen presenting cells are autologous to the patient, that is, the patient into which the sensitized APCs are injected is the patient from which the APC were originally isolated,.

Optionally, the ability of sensitized APC to stimulate, for example, the antigen-specific, class I-restricted cytotoxic T-lymphocytes (CTL) can be monitored by their ability to stimulate CTLs to release tumor necrosis factor, and by their ability to act as targets of such CTLs.

4.4.2.2 Reinfusion of Sensitized APC

The hsp–antigenic molecule-sensitized APC are reinfused into the patient systemically, preferably intravenously, by conventional clinical procedures. These activated cells are reinfused, preferentially by systemic administration into the autologous patient. Patients generally receive from about 10^6 to about 10^{12} sensitized macrophages, depending on the condition of the patient. In some regimens, patients may optionally receive in addition a suitable dosage of a biological response modifier including but not limited to the cytokines IFN-α, IFN-γ, IL-2, IL-4, IL-6, TNF or other cytokine growth factor.

4.4.3 Passive Immunotherapy

Hsp-ND associated antigen complexes can also be used for passive immunotherapy against neurodegenerative disorders. Passive immunity is the short-term protection of a host, achieved by the administration of pre-formed antibody directed against a heterologous organism. For example, hsp-ND associated antigen complexes may used to elicit an immune response in a subject, the sera removed from the subject and used for treatment or prevention of a neurodegerative disorder in a subject having a disorder caused by the presence of a common antigen.

4.5 Determination of Immunogenicity of Hsp– ND-Associated Antigen Complexes

Optionally, the heat shock protein ND-associated antigen complexes can be assayed for immunogenicity using any method known in the art. By way of example but not limitation, one of the following three procedures can be used.

4.5.1 MLTC Assay

Briefly, mice are injected with the hsp ND-associated antigen complex, using any convenient route of administration. As a negative control, other mice are injected with heat shock protein peptide complexes not associated with ND-associated antigens, or cells containing heat shock protein peptide complexes not associated with ND-associated antigens. Cells containing ND-associated antigens may act as a positive control for the assay. The mice are injected twice, 7-10 days apart. Ten days after the last immunization, the spleens are removed and the lymphocytes released. The released lymphocytes may be re-stimulated subsequently *in vitro* by the addition of dead cells that expressed the antigen of interest.

For example, $8x10^6$ immune spleen cells may be stimulated with $4x10^4$ mitomycin C treated or γ-irradiated (5-10,000 rads) cells containing the antigen of interest (or cells transfected with an appropriate gene, as the case may be) in 3ml RPMI medium containing 10% fetal calf serum. In certain cases 33% secondary mixed lymphocyte culture supernatant may be included in the culture medium as a source of T cell growth factors (*See,* Glasebrook, et al., 1980, *J. Exp. Med.* 151:876). To test the primary cytotoxic T cell response after immunization, spleen cells may be cultured without stimulation. In some experiments spleen cells of the immunized mice may also be re-stimulated with antigenically distinct cells, to determine the specificity of the cytotoxic T cell response.

Six days later the cultures are tested for cytotoxicity in a 4 hour ^{51}Cr-release assay (*see* Palladino *et al.*, 1987, *Cancer Res.* 47:5074-5079 and Blachere, at al., 1993, *J. Immunotherapy* 14:352-356). In this assay, the mixed lymphocyte culture is added to a target cell suspension to give different effector:target (E:T) ratios (usually 1:1 to 40:1). The target cells are prelabelled by incubating $1x10^6$ target cells in culture medium containing 20 mCi ^{51}Cr/ml for one hour at 37°C. The cells are washed three times following labeling. Each assay point (E:T ratio) is performed in triplicate and the appropriate controls incorporated to measure spontaneous ^{51}Cr release (no lymphocytes added to assay) and 100% release (cells lysed with detergent). After incubating the cell mixtures for 4 hours, the cells are pelletted by centrifugation at 200g for 5 minutes. The amount of ^{51}Cr released into the supernatant is measured by a gamma counter. The percent cytotoxicity is measured as cpm in the test sample minus spontaneously released cpm divided by the total detergent released cpm minus spontaneously released cpm.

In order to block the MHC class I cascade a concentrated hybridoma supernatant derived from K-44 hybridoma cells (an anti-MHC class I hybridoma) is added to the test samples to a final concentration of 12.5%.

4.5.2 CD4+ T Cell Proliferation Assay

Primary T cells are obtained from spleen, fresh blood, or CSF and purified by centrifugation using FICOLL-PAQUE PLUS (Pharmacia, Upsalla, Sweden) essentially as described by Kruse and Sebald, 1992, EMBO J. 11: 3237-3244. The peripheral blood mononuclear cells are incubated for 7-10 days with a lysate of cells expressing an ND-associated antigen. Antigen presenting cells may, optionally be added to the culture 24 to 48 hours prior to the assay, in order to process and present the antigen in the lysate. The cells are then harvested by centrifugation, and washed in RPMI 1640 media (GibcoBRL, Gaithersburg, Md.). $5x10^4$ activated T cells/well (PHA-blasts) are in RPMI 1640 media containing 10% fetal bovine serum, 10 mM HEPES, pH 7.5, 2 mM L-glutamine, 100 units/ml penicillin G, and 100 μg/ml streptomycin sulphate in 96 well plates for 72 hrs at 37°C., pulsed with 1 μCi ^{3}H-thymidine (DuPont NEN, Boston, Mass.)/well for 6 hrs, harvested, and radioactivity measured in a TOPCOUNT scintillation counter (Packard Instrument Co., Meriden, Conn.).

4.5.3 Antibody Response Assay

In one embodiment of the invention, the immunogenicity of an hsp ND-associated antigen complex is determined by measuring antibodies produced in response to the vaccination with the complex, by an antibody response assay, such as an enzyme-linked immunosorbent assay (ELISA) assay. Methods for such assays are well known in the art (see, *e.g.*, Section 2.1 of Current Protocols in Immunology, Coligan *et al.* (eds.), John Wiley and Sons, Inc. 1997). In one mode of the embodiment, microtitre plates (96-well Immuno Plate II, Nunc) are coated with 50 μl/well of a 0.75 μg/ml solution of a purified, non-hsp-complexed form of the ND-associated antigen used in the vaccine (*e.g.* Aβ42) in PBS at 4°C for 16 hours and at 20°C for 1 hour. The wells are emptied and blocked with 200 μl PBS-T-BSA (PBS containing 0.05% (v/v) TWEEN 20 and 1% (w/v) bovine serum albumin) per well at 20°C for 1 hour, then washed 3 times with PBS-T. Fifty μl/well of plasma or CSF from a vaccinated animal (such as a model mouse or a human patient) is applied at 20°C for 1 hour, and the plates are washed 3 times with PBS-T. The anti-ND associated antigen antibody activity is then measured calorimetrically after incubating at 20°C for 1 hour with 50μl/well of sheep anti-mouse or anti-human immunoglobulin, as appropriate, conjugated with horseradish peroxidase (Amersham) diluted 1:1,500 in PBS-T-BSA and (after 3 further PBS-T washes as above) with 50 μl of an o-phenylene diamine (OPD)-H_2O_2 substrate solution. The reaction is stopped with 150 μl of 2M H_2SO_4 after 5 minutes and absorbance is determined in a Kontron SLT-210 photometer (SLT Lab-instr., Zurich, Switzerland) at 492 nm (ref. 620 nm).

4.6 Methods for Diagnosing and Assaying Progress of Neurodegenerative Disorders, Such as AD

There are presently many molecular markers known in the art for the diagnosis of neurodegenerative disorders, such as AD (see, *e.g.*, Bancher et al., 1998, J. Neural Transm., Suppl. 53:185-197; Galasko, 1998, J. Neural Transm., Suppl. 53:209-221; issue no. 2 of Neurobiol. Aging, Vol. 19, 1998, including articles by Arai et al., pp. 125-6; Foster, pp. 127-129; Mayeux, pp.139-143; Klunk, pp. 145-157; Hock, pp. 149-151; Robles, pp. 153-157; Hyman, pp. 159-160; and Lannfelt, pp. 165-167). These methods can be used to determine whether an asymptomatic human subject displays any of the molecular hallmarks of a specific neurodegenerative disorder. The methods may also be used to diagnose a neurodegenerative disorder in a human subject who exhibits symptoms of the early stages of the neurodegenerative disorder. These methods are useful for identifying individuals at risk of a neurodegenerative disorder who would benefit from the methods of the invention of treatment and prevention of neurodegenerative disorders. Finally, the methods can also be used to assay the efficacy of the vaccines of the present invention and monitor the progress of a neurodegenerative disorder in those receiving the vaccines. The diagnostic methods to be utilized according to the present invention include but are not limited to testing for molecular indicators of a particular neurodegenerative disorder, such as AD, or for alterations in neurophysiological function that would be affected by such a neurodegenerative disorder. In a preferred embodiment, more than one of the assays described below would be carried out to confirm the diagnosis of the presence or the extent of progession of the neurodegenerative disorder.

4.6.1 Molecular Indicators of Neurodegenerative Disorders

Testing for the presence of phosphorylated tau protein using a monoclonal antibody specific for the phosphorylated form, as disclosed in U.S. Patent No. 5,733,734, serves as an indicator for neurodegenerative disorders, such as AD. In a preferred embodiment, the monoclonal antibody is Alz-50 (U.S. Patent No. 5,811,310). The presence of phosphorylated Tau may be tested in either brain tissue or cerebrospinal fluid or cultures of olfactory neurons from the patient.

Tau proteolysis products have been found to be present in the blood or spinal fluids of individuals with AD (U.S. Patent No. 5,492,812). Thus, testing for the presence of tau peptides in blood or spinal fluid samples may provide a diagnostic measure of the presence or progression of AD in patients and other individuals.

The presence or extent of AD can also be determined by measuring the relative abundance of Aβ42 and Aβ40. In normal individuals, the amount of Aβ40 far exceeds the amount of Aβ42. In contrast, Aβ42 predominates in AD patients.

Additionally, all mutations implicated in FAD, whether in the *APP*, *PS1*, or *PS2* genes, relate to the processing of APP, and are thought to produce AD through promoting the synthesis of Aβ42. Thus, the relative amount of Aβ40 and Aβ42, for example in a CSF sample or tissue biopsy from the brain or pancreas from an individual, would be an indicator of the presence or progress of AD in the individual.

Calcium activated neutral proteases are enzymes that regulate signal transduction by modulating the activities of signaling molecules (*e.g.* protein kinases and phosphatases) through partial proteolysis. Calcium activated neutral proteases are themselves regulated by partial proteolysis, wherein in the presence of calcium a precursor form of an enzyme undergoes autoproteolytic cleavage to produce a functional enzyme. It has been shown that the ratios of cleaved to uncleaved calcium activated neutral proteases are altered in AD patients. Thus, by measuring the relative amounts of each isoform in a test subject in comparison with a control subject, it is possible to detect AD in an individual (U.S. Patent No. 5,624,807).

It has been demonstrated that patients with neurodegenerative disorders, such as AD, have elevated levels of acetylcholinesterase (AChE) activity in ocular fluids. A colorimetric assay for the determination of AChE activity described by Ellman et al. (1961, Biochem. Pharmacol. 7:161-177) may be utilized to measure AchE activity levels in ocular fluid samples, *i.e.* aqueous humor or vitreous humor samples, the result of which would indicate the presence or absence and possibly the extent of AD (U.S. Patent No. 5,595,883).

One of the characteristics of neurodegenerative disorders, such as AD, is an impairment in cytokine secretion, for example IL-1, IL-3 and IL-6. It has been postulated that the impairment is a downstream effect of impaired neural function. Thus it would be possible to assay for blood cytokine levels as indicators of AD (U.S. Patent No. 5,874,312)

Unless indicated otherwise, the proteins or peptides described *supra* may be assayed for by a radioimmunoassay, an enzyme-linked immunosorbant assay (ELISA), a sandwich assay, a gel immunodiffusion assay, an agglutination assay, a fluorescent immunoassay, a protein A immunoassay or an immunoelectrophoresis assay, or any other method known in the art. These methods are well known to those skilled in the art.

4.6.2 Neurophysiological Tests

U.S. Patent No.5,778,893 discloses methods of diagnosing AD, including the extent of AD, in an individual by applying agents that agonize or antagonize neuromuscular signaling and determining the response of the individual to said agents in comparison to control individuals. For example, a cholinergic antagonist is administered to the eye of an individual, the pupil allowed to dilate in response to the cholinergic antagonist, and the rate

of which the pupil returns to its normal diameter measured and compared to the corresponding rates in control individuals.

In an alternative method of measuring neurophysiological output, a light source illuminates the eye of an individual suspected to have AD. The response of the individual's pupils to the light is measured by a computer system connected to a video camera that records the response of the pupils (U.S. Patent 5,883,691).

4.7 Dosage Regimens

Dosages of hsp- ND-associated antigen complexes can be extrapolated from prior art methods established in experimental tumor models (Blachere *et al.*, 1993, J. Immunotherapy 14:352-356). Extrapolation to human dosages of based on body weight and surface area. For example, prior art methods of extrapolating human dosage based on body weight can be carried out as follows: since the conversion factor for converting the mouse dosage to human dosage is Dose Human per kg = Dose Mouse per kg x 12 (*See* Freireich, E.J., *et al.*, 1966, Cancer Chemotherap. Rep. 50: 219-244), the effective dose of HSP-peptide complexes in humans weighing 70kg should be 1mg/kg ÷ 12 x 70, *i.e.*, about 6mg (5.8mg).

Drug doses are also given in milligrams per square meter of body surface area because this method rather than body weight achieves a good correlation to certain metabolic and excretionary functions (Shirkey, 1965, JAMA 193: 443). Moreover, body surface area can be used as a common denominator for drug dosage in adults and children as well as in different animal species as indicated below in Table 1 (Freireich *et al.*, 1966, Cancer Chemotherap. Rep. 50: 219-244).

TABLE 1

REPRESENTATIVE SURFACE AREA TO WEIGHT RATIOS (km) FOR VARIOUS SPECIES[1]

Species	Body Weight (kg)	Surface Area (Sqm)	km Factor
Mouse	0.02	0.0066	3.0
Rat	0.15	0.025	5.9
Monkey	3.0	0.24	12
Dog	8.0	0.40	20
Human, Child	20	0.80	25
Adult	60	1.6	37

Example: To express a mg/kg dose in any given species as the equivalent mg/sq m dose, multiply the dose by the appropriate km factor. In adult human, 100mg/kg is equivalent to 100 mg/kg x 37 kg/sq m = 3700 mg/sq m.

In contrast to both of the above-described prior art methods of determining dosage levels, the present invention provides dosages of the purified complexes of hsps and ND-associated antigenic molecules that are much smaller than the dosages estimated by the prior art. For example, according to the invention, an amount of Hsp70– ND-associated antigenic molecule complexes and/or gp96 ND-associated –antigenic molecule complexes is administered that is in the range of about 2 microgram to about 5000 micrograms for a human patient, the preferred human dosage being the same as used in a 25g mouse. The dosage for Hsp-90 peptide complexes in a human patient provided by the present invention is in the range of about 10 to 1,000 micrograms, the preferred dosage being 20 micrograms.

The doses recited above are preferably given once weekly for a period of about 4-6 weeks, and the mode or site of administration is preferably varied with each administration. In a preferred example, intradermal administrations are given, with each site of administration varied sequentially. Thus, by way of example and not limitation, the first injection may be given subcutaneously on the left arm, the second on the right arm, the third on the left belly, the fourth on the right belly, the fifth on the left thigh, the sixth on the right thigh, *etc.* The same site may be repeated after a gap of one or more injections. Also,

[1] Freireich, *et al.*, 1966, Cancer Chemotherap. Rep. 50: 219-244.

split injections may be given. Thus, for example, half the dose may be given in one site and the other half on an other site on the same day.

Alternatively, the mode of administration is sequentially varied, *e.g.*, weekly injections are given in sequence intradermally, intramuscularly, intravenously or intraperitoneally.

After 4-6 weeks, further injections are preferably given at two-week intervals over a period of time of one month. Later injections may be given monthly. The pace of later injections may be modified, depending upon the patient's clinical progress and responsiveness to the immunotherapy.

4.8 Vaccine Formulation

Complexes of hsps and ND-associated antigenic molecules purified by the methods of the invention may be formulated into pharmaceutical preparations for administration to mammals for treatment or prevention of neurodegenerative disorders and diseases. Drug solubility and the site of absorption are factors which should be considered when choosing the route of administration of a therapeutic agent. Hsp-ND-associated antigenic molecule complexes of the invention may be administered using any desired route of administration, including but not limited to, *e.g.*, intradermally, subcutaneously, intravenously or intramuscularly, although intradermally is preferred. Advantages of intradermal administration include use of lower doses and rapid absorption, respectively. Mucosal routes of administration include, but are not limited to, oral, rectal and nasal administration. Preparations for mucosal administrations are suitable in various formulations as described below. The route of administration can be varied during a course of treatment. Preferred dosages, routes of administration and therapeutic regimens for complexes of peptides and naturally occurring HSPs are described in PCT International patent applications published as WO 96/10411 and WO 97/10001, which are incorporated by reference herein in their entireties.

Compositions comprising hsp-ND-associated antigenic complexes formulated in a compatible pharmaceutical carrier may be prepared, packaged, and labeled for treatment of the indicated neurodegenerative disorder. In preferred aspects, an amount of hsp-ND-associated antigenic complex is administered to a human that is in the range of about 2 to 150 μg, preferably 20 to 20 μg, most preferably about 5 μg, given once weekly for about 4-6 weeks, intradermally with the site of administration varied sequentially.

If the complex is water-soluble, then it may be formulated in an appropriate buffer, for example, phosphate buffered saline or other physiologically compatible solutions. Alternatively, if the resulting complex has poor solubility in aqueous solvents, then it may be formulated with a non-ionic surfactant such as Tween, or polyethylene

glycol. Thus, the hsp complexes and their physiologically acceptable solvates may be formulated for administration by inhalation or insufflation (either through the mouth or the nose) or oral, buccal, parenteral, rectal administration.

For oral administration, the pharmaceutical preparation may be in liquid form, for example, solutions, syrups or suspensions, or may be presented as a drug product for reconstitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (*e.g.*, sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (*e.g.*, lecithin or acacia); non-aqueous vehicles (*e.g.*, almond oil, oily esters, or fractionated vegetable oils); and preservatives (*e.g.*, methyl or propyl-p-hydroxybenzoates or sorbic acid). The pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (*e.g.*, pregelatinized maize starch, polyvinyl pyrrolidone or hydroxypropyl methylcellulose); fillers (*e.g.*, lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (*e.g.*, magnesium stearate, talc or silica); disintegrants (*e.g.*, potato starch or sodium starch glycolate); or wetting agents (*e.g.*, sodium lauryl sulphate). The tablets may be coated by methods well-known in the art.

Preparations for oral administration may be suitably formulated to give controlled release of the complexes. Such compositions may take the form of tablets or lozenges formulated in conventional manner.

In a specific embodiment, the hsp compositions of the present invention are administered intrathecally by an implant be placed in or near the lesioned area of the nervous system. Suitable implants include, but are not limited to, gelfoam, wax, liposome or microparticle-based implants. Such compositions are preferably used when it is desired to achieve sustained release of the hsp-peptide complexes.

For administration by inhalation, the complexes may be conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, *e.g.*, gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the complexes and a suitable powder base such as lactose or starch.

The complexes may be formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, *e.g.*, in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions

in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

The complexes may also be formulated in rectal compositions such as suppositories or retention enemas, *e.g.*, containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the complexes may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example, subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the complexes may be formulated with suitable polymeric or hydrophobic materials (for example, as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt. Liposomes and emulsions are well known examples of delivery vehicles or carriers for hydrophilic drugs.

The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. Compositions comprising a compound of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition.

The invention also provides kits for carrying out the therapeutic regimens of the invention. Such kits comprise in one or more containers therapeutically or prophylactically effective amounts of hsp-ND associated peptide complexes in pharmaceutically acceptable form. The hsp-ND associated peptide complexes in a vial of a kit of the invention may be in the form of a pharmaceutically acceptable solution, *e.g.*, in combination with sterile saline, dextrose solution, or buffered solution, or other pharmaceutically acceptable sterile fluid. Alternatively, the complex may be lyophilized or desiccated; in this instance, the kit optionally further comprises in a container a pharmaceutically acceptable solution (*e.g.*, saline, dextrose solution, *etc.*), preferably sterile, to reconstitute the complex to form a solution for injection purposes.

In another embodiment, a kit of the invention further comprises a needle or syringe, preferably packaged in sterile form, for injecting the complex, and/or a packaged alcohol pad. Instructions are optionally included for administration of hsp – ND-associated antigen complexes by a clinician or by the patient.

4.8.1 Determination of Vaccine Efficacy

The immunopotency of ND-associated antigens can be determined by monitoring the immune response in test animals following immunization with the ND-associated antigen, or by use of any immunoassay known in the art. Generation of a humoral (antibody) response and/or cell-mediated immunity, may be taken as an indication of an immune response. Test animals may include mice, hamsters, dogs, cats, monkeys, rabbits, chimpanzees, *etc.*, and eventually human subjects.

Methods of introducing the vaccine may include oral, intracerebral, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal or any other standard routes of immunization. The immune response of the test subjects can be analyzed by various approaches such as: the reactivity of the resultant immune serum to the neurodegenerative disorder antigen, as assayed by known techniques, *e.g.*, immunosorbant assay (ELISA), immunoblots, radioimmunoprecipitations, *etc.*, or by protection of the immunized host against the neurodegenerative disorder.

As one example of suitable animal testing of a vaccine protective against neurodegenerative disorders and diseases, the vaccine of the invention may be tested in rabbits for the ability to induce an antibody response to the ND-associated antigen. Male specific-pathogen-free (SPF) young adult New Zealand White rabbits may be used. The test group each receives a fixed concentration of the vaccine. A control group receives an injection of 1 mM Tris-HCl pH 9.0 without the ND-associated antigen.

Blood samples may be drawn from the rabbits every one or two weeks, and serum analyzed for antibodies to the ND-associated protein. The presence of antibodies specific for the antigen may be assayed, *e.g.*, using an ELISA assay.

4.8.2 Monitoring of Effects During Immunotherapy

The effect of immunotherapy with hsp-ND-associated antigenic complexes on progression of neurodegerative diseases can be monitored by any methods known to one skilled in the art. In addition, cellular immunity may be monitored by methods including but not limited to measuring: a) delayed hypersensitivity as an assessment of cellular immunity; b) activity of cytolytic T-lymphocytes *in vitro*; c) levels of ND-associated antigen, *e.g.*, 42/43 β-amyloid.

Delayed hypersensitivity skin tests are also of great value in the overall immunocompetence and cellular immunity to an antigen. Inability to react to a battery of common skin antigens is termed anergy (Sato *et al.*, 1995, Clin. Immunol. Pathol. 74: 35-43).

Proper technique of skin testing requires that the antigens be stored sterile at 4°C, protected from light and reconstituted shorted before use. A 25- or 27-gauge needle ensures intradermal, rather than subcutaneous, administration of antigen. Twenty-four and

48 hours after intradermal administration of the antigen, the largest dimensions of both erythema and induration are measured with a ruler. Hypoactivity to any given antigen or group of antigens is confirmed by testing with higher concentrations of antigen or, in ambiguous circumstances, by a repeat test with an intermediate test.

In another optional method, the activity of cytolytic T-lymphocytes can be assessed *in vitro* using the following method. Eight x 10^6 peripheral blood-derived T lymphocytes isolated by the Ficoll-Hypaque centrifugation gradient technique, are restimulated with $4x10^4$ mitomycinC-treated cells in 3ml RPMI medium containing 10% fetal calf serum. In some experiments, 33% secondary mixed lymphocyte culture supernatant or IL-2, is included in the culture medium as a source of T cell growth factors.

In order to measure the primary response of cytolytic T-lymphocytes after immunization, T cells are cultured without the stimulator cells. In other experiments, T cells are restimulated with antigenically distinct cells. After six days, the cultures are tested for cytotoxicity in a 4 hour ^{51}Cr-release assay. The spontaneous ^{51}Cr-release of the targets should reach a level less than 20%. For the anti-MHC class I blocking activity, a tenfold concentrated supernatant of W6/32 hybridoma is added to the test at a final concentration of 12.5% (Heike *et al.*, J. Immunotherapy 15: 165-174).

In immunization procedures, the amount of immunogen to be used and the immunization schedule will be determined by a physician skilled in the art and will be administered by reference to the immune response and antibody titers of the subject.

The invention is not to be limited in scope by the specific embodiments described which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

All references cited herein, including patent applications, patents, and other publications, are incorporated by reference herein in their entireties for all purposes.

WHAT IS CLAIMED IS:

1. A purified complex comprising a heat shock protein and an antigenic molecule, which antigenic molecule displays the antigenicity of an antigen associated with a neurodegenerative disorder.

2. The complex of Claim 1 which is the product of a method comprising complexing said heat shock protein and said antigenic molecule in vitro.

3. The complex of Claim 1 in which the complex is a fusion protein comprising the heat shock protein fused to the antigenic molecule.

4. The complex of Claim 1 in which the heat shock protein is Hsp70, Hsp90, gp96, PDI, or calreticulin.

5. The complex of Claim 1 in which the heat shock protein is noncovalently associated with the antigenic molecule.

6. The complex of Claim 1, wherein the complex of a heat shock protein and an antigenic molecule is purified to apparent homogeneity, as viewed on an SDS-PAGE gel.

7. The complex of Claim 1 in which the antigenic molecule is β-amyloid or a fragment thereof, an oligomeric Aβ complex or a fragment thereof, an ApoE4-Aβ complex or a fragment thereof, tau protein or a fragment thereof, a mutant amyloid precurser protein or a fragment thereof, a mutant of presenillin or a fragment thereof, α-synuclein or a fragment thereof, or a prion protein or a fragment thereof.

8. The complex of Claim 7 in which the antigenic molecule is β-amyloid, or a fragment thereof.

9. The complex of Claim 1 in which the antigenic molecule is coupled to a T cell helper epitope.

10. The complex of Claim 9 in which the T cell helper epitope is tuberculin purified protein derivative, tetanus toxin sequences, or malarial parasite sequences.

11. A composition comprising a purified population of complexes of heat shock proteins bound to antigenic molecules, said complexes being purified from cells transformed with and expressing a nucleic acid encoding a protein displaying the antigenicity of an antigen associated with a neurodegenerative disorder.

12. The composition of Claim 11, wherein the population of complexes of heat shock proteins bound to antigenic molecules is purified to apparent homogeneity, as viewed on an SDS-PAGE gel.

13. A pharmaceutical composition comprising an amount of a purified molecular complex effective for treatment or prevention of a neurodegenerative disorder, and a pharmaceutically acceptable carrier, said molecular complex comprising a heat shock protein and an antigenic molecule, which antigenic molecule displays the antigenicity of an antigen associated with a neurodegenerative disorder.

14. The pharmaceutical composition of Claim 13 in which the heat shock protein is covalently associated with the antigenic molecule.

15. The pharmaceutical composition of Claim 13 in which the antigenic molecule is coupled to a T cell helper epitope.

16. The complex of Claim 15 in which the T cell helper epitope is tuberculin purified protein derivative, tetanus toxin sequences, or malarial parasite sequences.

17. The pharmaceutical composition of Claim 13 wherein the heat shock protein is Hsp70, Hsp90, gp96, PDI, or calreticulin.

18. The pharmaceutical composition of Claim 13 in which the antigenic molecule is β-amyloid or a fragment thereof, an oligomeric Aβ complex or a fragment thereof, an ApoE4-Aβ complex or a fragment thereof, tau protein or a fragment thereof, a mutant amyloid precurser protein or a fragment thereof, a mutant of presenillin or a fragment thereof, α-synuclein or a fragment thereof, or a prion protein or a fragment thereof.

19. The pharmaceutical composition of Claim 13 wherein the neurodegenerative disorder is Alzheimer's Disease, age-related loss of cognitive function, senile dementia, Parkinson's disease, amyotrophic lateral sclerosis, Wilson's Disease, cerebral palsy,

progressive supranuclear palsy, Guam disease, Lewy body dementia, prion diseases, spongiform encephalopathies, Creutzfeldt-Jakob disease, polyglutamine diseases, Huntington's disease, myotonic dystrophy, Freidrich's ataxia, ataxia, Gilles de la Tourette's syndrome, seizure disorders, epilepsy, chronic seizure disorder, stroke, brain trauma, spinal cord trauma, AIDS dementia, alcoholism, autism, retinal ischemia, glaucoma, autonomic function disorder, hypertension, neuropsychiatric disorder, schizophrenia, or schizoaffective disorder.

20. The pharmaceutical composition of Claim 15 wherein the neurodegenerative disorder is Alzheimer's Disease.

21. A recombinant cell transformed with (i) a first nucleic acid comprising a first nucleotide sequence that is operably linked to a first promoter and that encodes a heat shock protein, and (ii) a second nucleic acid comprising a second nucleotide sequence that is operably linked to a second promoter and encodes an antigenic molecule that displays the antigenicity of an antigen associated with a neurodegenerative disorder, such that the heat shock protein and the antigenic molecule are expressed within the cell and non-covalently associate with each other to form a complex that in sufficient amount is capable of eliciting an immune response to the antigenic molecule.

22. The recombinant cell of Claim 21 wherein the cell is a human cell.

23. A recombinant cell transformed with a nucleic acid comprising a nucleic acid sequence that is operably linked to a promoter, said nucleic acid sequence encoding a fusion protein that comprises a heat shock protein operatively linked to an antigenic molecule that displays the antigenicity of an antigen associated with a neurodegenerative disorder.

24. The recombinant cell of Claim 21 or 23, wherein the antigenic molecule is β-amyloid or a fragment thereof, an oligomeric Aβ complex or a fragment thereof, an ApoE4-Aβ complex or a fragment thereof, tau protein or a fragment thereof, a mutant amyloid precurser protein or a fragment thereof, a mutant of presenillin or a fragment thereof, α-synuclein or a fragment thereof, or a prion protein or a fragment thereof.

25. The recombinant cell of Claim 24, wherein the antigenic molecule is β-amyloid, or a fragment thereof.

26. A pharmaceutical composition comprising the recombinant cell of Claim 21 or 19 and a pharmaceutically acceptable carrier.

27. A method for preparing a complex of a heat shock protein associated with an antigenic molecule that displays the antigenicity of an antigen associated with a neurodegenerative disorder, comprising:

a) culturing a recombinant cell that expresses a heat shock protein and is transformed with a nucleic acid comprising a nucleotide sequence that is operably linked to a promoter and encodes an antigenic molecule that displays the antigenicity of an antigen associated with a neurodegenerative disorder, under conditions such that the antigenic molecule is expressed by the cell and associates with the heat shock protein expressed by the cell; and

b) recovering a population of complexes of the heat shock proteins noncovalently associated with the antigenic molecules.

28. A method for preparing a complex of a heat shock protein associated with an antigenic molecule that displays the antigenicity of an antigen associated with a neurodegenerative disorder, comprising:

a) culturing a recombinant cell transformed with (i) a first nucleic acid comprising a first nucleotide sequence that is operably linked to a first promoter and that encodes a heat shock protein, and (ii) a second nucleic acid comprising a second nucleotide sequence that is operably linked to a second promoter and encodes an antigenic molecule that displays the antigenicity of an antigen associated with a neurodegenerative disorder, under conditions such that the heat shock protein is expressed by the cell and associates with the antigenic molecule; and

b) recovering a population of complexes of the heat shock protein noncovalently associated with antigenic molecules from the cells.

29. The method of Claim 27 or 28, further comprising the step of c) purifying the complexes.

30. The method of Claim 29, wherein the complexes are purified by affinity chromatography.

31. The method of Claim 29, further comprising the step of d) treating the purified complexes with a crosslinking agent such that the hsps become covalently attached to the antigenic molecules.

32. A method for preparing a fusion protein capable of eliciting an immune response against a neurodegenerative disorder, said method comprising:

a) culturing a recombinant cell transformed with a nucleic acid comprising a nucleotide sequence that is operably linked to a promoter and that encodes a fusion protein comprising a heat shock protein operatively linked to an antigenic molecule that displays the antigenicity of an antigen associated with a neurodegenerative disorder, under conditions such that the fusion protein is expressed by the cell; and

b) recovering the fusion protein from the cells.

33. A method for preparing *in vitro* a complex of a heat shock protein associated with one or more antigenic molecules that display the antigenicities of antigens associated with a neurodegenerative disorder, said method comprising:

a) incubating a heat shock protein and the antigenic molecule or molecules under conditions and for a length of time sufficient for formation of the complex, and

b) isolating said complexes.

34. The method of Claim 33, wherein the heat shock protein is purified.

35. The method of Claim 33, further comprising the step of c) treating the isolated complex with a crosslinking agent such that the heat shock protein of the complex becomes covalently attached to one or more antigenic molecules of the complex.

36. The method of Claim 29, 31, 32, or 33, wherein the one or more antigenic molecules is β-amyloid or a fragment thereof, an oligomeric Aβ complex or a fragment thereof, an ApoE4-Aβ complex or a fragment thereof, tau protein or a fragment thereof, a mutant amyloid precurser protein or a fragment thereof, a mutant of presenillin or a fragment thereof, α-synuclein or a fragment thereof, or a prion protein or a fragment thereof.

37. The method of Claim 36, wherein one or more of the antigenic molecules is β-amyloid, or a fragment thereof.

38. A method for eliciting an immune response against an antigen associated with a neurodegenerative disorder in an individual comprising administering to the individual a complex of a heat shock protein and an antigenic molecule that displays the antigenicity of an antigen associated with a neurodegenerative disorder in an amount effective to elicit an immune response.

39. The method of Claim 38, further comprising, before, concurrently, or after administration of the immunogenic complex, administering to the individual a composition comprising antigen presenting cells sensitized *in vitro* with a sensitizing amount of a second complex of a heat shock protein and a second antigenic molecule, in which said second antigenic molecule shares at least one antigenic determinant with the first antigenic molecule.

40. A method of treating or protecting against a neurodegenerative disorder in an individual having a neurodegenerative disorder, or in whom prevention of a neurodegenerative disorder is desired, comprising administering to the individual a purified complex of a heat shock protein and an antigenic molecule in an amount effective to treat or protect against said neurodegenerative disorder, wherein said antigenic molecule displays the antigenicity of an antigen associated with said neurodegenerative disorder.

41. The method of Claim 40, further comprising, before, concurrently or after administration of the immunogenic complex, administering to the individual a composition comprising antigen presenting cells sensitized *in vitro* with a sensitizing amount of a second complex of a heat shock protein and a second antigenic molecule, said second antigenic molecule sharing at least one antigenic determinant with the first antigenic molecule.

42. A method of treating or protecting against a neurodegenerative disorder in a subject having a neurodegenerative disorder or in whom prevention of a neurodegenerative disorder is desired comprising:

a) culturing a recombinant cell that expresses a heat shock protein and is transformed with a nucleic acid comprising a nucleotide sequence that is operably linked to a promoter and encodes an antigenic molecule that displays the antigenicity of an antigen associated with a neurodegenerative disorder, under conditions such that the antigenic

molecule is expressed by the cell and associates with the heat shock protein expressed by the cell; and

b) recovering a population of complexes of the heat shock proteins noncovalently associated with the antigenic molecules; and

c) administering to the subject an amount of the recovered complexes effective to treat or protect against the neurodegenerative disorder.

43. A method of treating or protecting against a neurodegenerative disorder in a subject having a neurodegenerative disorder or in whom prevention of a neurodegenerative disorder is desired comprising:

a) culturing a recombinant cell transformed with (i) a first nucleic acid encoding a heat shock protein, and (ii) a second nucleic acid encoding an antigenic molecule displaying the antigenicity of an antigen associated with a neurodegenerative disorder;

b) recovering complexes of the heat shock protein noncovalently associated with the antigenic molecule; and

c) administering to the subject an amount of the recovered complexes effective to treat or protect against the neurodegenerative disorder.

44. The method of Claim 42 or 43, further comprising, after step (b) and before step (c), the step of treating the complexes with a crosslinking agent such that the heat shock protein becomes covalently associated with the antigenic molecule.

45. A method of treating or protecting against a neurodegenerative disorder in a subject having a neurodegenerative disorder or in whom prevention of a neurodegenerative disorder is desired comprising:

a) culturing a recombinant cell transformed with a nucleic acid comprising a nucleotide sequence that is operably linked to a promoter and that encodes a fusion protein comprising a heat shock protein operatively linked to an antigenic molecule that displays the antigenicity of an antigen associated with a neurodegenerative disorder, under conditions such that the fusion protein is expressed by the cell;

b) recovering the fusion protein from the cells; and

c) administering to the subject an amount of the fusion protein effective to treat or protect against the neurodegenerative disorder.

46. The method of any one of Claims 38, 40, 42, 43, 44, or 45, wherein the neurodegenerative disorder is Alzheimer's Disease, age-related loss of cognitive function, senile dementia, Parkinson's disease, amyotrophic lateral sclerosis, Wilson's Disease, cerebral palsy, progressive supranuclear palsy, Guam disease, Lewy body dementia, prion diseases, spongiform encephalopathies, Creutzfeldt-Jakob disease, polyglutamine diseases, Huntington's disease, myotonic dystrophy, Freidrich's ataxia, ataxia, Gilles de la Tourette's syndrome, seizure disorders, epilepsy, chronic seizure disorder, stroke, brain trauma, spinal cord trauma, AIDS dementia, alcoholism, autism, retinal ischemia, glaucoma, autonomic function disorder, hypertension, neuropsychiatric disorder, schizophrenia, or schizoaffective disorder.

47. The method of Claim 46, wherein the neurodegenerative disorder is Alzheimer's Disease.

48. The method of Claim 46, wherein the antigenic molecule is a β-amyloid or a fragment thereof, an oligomeric Aβ complex or a fragment thereof, an ApoE4-Aβ complex or a fragment thereof, tau protein or a fragment thereof, a mutant amyloid precurser protein or a fragment thereof, a mutant of presenillin or a fragment thereof, α-synuclein or a fragment thereof, or a prion protein or a fragment thereof.

49. The method of Claim 48, wherein the antigenic molecule is a β-amyloid or a fragment thereof.

50. The method of any one of Claims 38, 40, 42, 43, 44, or 45, wherein the antigenic molecule is coupled to a T cell helper epitope.

51. The complex of Claim 50, in which the T cell helper epitope is tuberculin purified protein derivative, tetanus toxin sequences, or malarial parasite sequences.

SEQUENCE LISTING

<110> University of Connecticut Health Care Center

<120> HEAT SHOCK/STRESS PROTEIN COMPLEXES AS VACCINES AGAINST NEURODEGENERATIVE DISORDERS

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/01825

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) :A61K 39/395, 39/40, 39/42, 39/44, 39/00, 39/385
US CL :424/180.1, 185.1, 193.1, 198.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. *FIELDS SEARCHED*

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/180.1, 185.1, 193.1, 198.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, MEDLINE, CAPLUS, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BROWN, I.R. Stress Genes in the Nervous System During Development and Aging. Ann. N.Y. Acad. Sci. 1998, Vol. 851, pages 123-128, see entire document.	1-36
Y	US 5,958,919 A (OLNEY et al.) 28 September 1999, see entire document.	1-36
Y	US 5,851,996 A (KLINE) 22 December 1998, see entire document.	1-36
Y	US 5,840,540 A (ST. GEORGE-HYSLOP et al.) 24 November 1998, see entire document.	1-36

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 06 APRIL 2001	Date of mailing of the international search report 27 APR 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer GERALD R. EWOLDT Telephone No. (703) 308-0196

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